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The aroma of Portuguese wine grapes: impact of infection with *Botrytis cinerea*

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Resumo

As uvas são das frutas mais produzidas e comercializadas em Portugal. À escala mundial é um dos frutos mais importantes a nível económico. As uvas que provêm de castas de videira diferentes apresentam características próprias como por exemplo, a cor e o seu aroma (que é constituído por determinadas concentrações de compostos específicos). A Trincadeira é uma das castas mais produzidas em Portugal originando uvas que, mais tarde, podem produzir excelentes vinhos. No entanto, as videiras são suscetíveis a vários agentes patogénicos, sendo a Trincadeira muito sensível ao fungo *Botrytis cinerea*. Este fungo reduz drasticamente a colheita destruindo parcial ou completamente os cachos de uvas, alterando a sua qualidade e características biológicas. Ao alterar a qualidade das uvas, o seu aroma também sofre várias modificações. Deste modo, o conjunto de compostos voláteis libertados para a atmosfera correspondentes ao aroma vão ser diferentes do que é considerado adequado, prejudicando a produção do vinho feita a partir destas uvas. Esta tese de mestrado tem como objetivo dar a conhecer as mudanças que ocorrem no aroma das uvas da Trincadeira quando infetada pela *Botrytis cinerea*, que degrada todos os tecidos das uvas. Neste estudo foram utilizadas uvas no seu último estádio de desenvolvimento EL38, também denominado por *estádio de colheita*. O estudo visa a caracterização dos compostos voláteis livres e glicosilados responsáveis pelo aroma da uva Trincadeira, com e sem a infeção do fungo *B. cinerea*, e ainda apurar a quantidade de fenóis e antocianinas. Os genes (enzimas) responsáveis pela síntese dos voláteis livres e glicosilados também foram analisados de modo a verificar quais os que obtiveram uma maior modificação e nível de expressão, devido à infeção. A análise dos voláteis foi feita a partir da técnica Headspace solid-phase microextraction (HP-SPME) onde o sumo da uva foi aquecido em banho maria, os voláteis libertados e absorvidos para serem de seguida identificados por cromatografia gasosa e espectrometria de massa (GC- MS). Inicialmente foram determinados os voláteis livres e, só após esta análise se determinou os glicosilados com a adição da enzima *β*-glycosidase. Com base nos resultados deste estudo identificaram-se vários possíveis marcadores da infeção que surgiram face à presença do fungo *Botrytis cinerea* e também, por parte do aceleramento da maturação do sumo de uva pela enzima *β*-glycosidase. Com base nestes voláteis identificados na Trincadeira e na bibliografia, selecionaram-se os vários genes que codificam para enzimas envolvidas na síntese de compostos voláteis específicos. Deste modo, procedeu-se à análise da expressão dos genes responsáveis pela origem do aroma por PCR quantitativo em tempo real (RT-PCR) para verificar se estes apresentavam um maior ou menor nível de expressão, em relação ao efeito produzido pelo fungo na sua síntese. No final, apenas os genes referentes aos voláteis livres, *hydroperoxide lyase 2* (*HPL2*) e *lipoxygenase C* (*LOXC*) apresentaram um maior nível de expressão quando as uvas se encontravam infetadas. A expressão dos genes referentes aos voláteis glicosilados, *glutathione synthetase 21 (GS21)* e *O-methyltransferase 1* (*OMT1*) também aumentaram ligeiramente nas uvas infetadas. Esta informação sugere que a infeção com o fungo *B. cinerea* modifica a expressão destes genes. O *HPL2* é expresso quando a uva sofre danos no tecido vegetal ou fica sujeito a stress tanto abiótico como biótico, uma vez que este gene regula o reconhecimento e a resistência tanto a insetos como a agentes patogénicos para mais tarde os eliminar. Com a infeção da *Botrytis* o *HPL2* aumenta a sua expressão na uva infectada. Em relação à enzima *LOXC*, sabe-se que é responsável pela produção de aldeídos e álcoois e o seu nível de expressão, por norma, decresce no último estádio de maturação da uva (*harvest* stage). No entanto, durante a infeção a sua expressão aumenta. A enzima GS21 é responsável pelo metabolismo dos aminoácidos e tem como função, prevenir a perda do aroma e o aparecimento de manchas castanhas na pele da uva. Face aos resultados, o nível de expressão nas uvas infetadas e nos controlos é muito semelhante, não se conseguindo obter resultados conclusivos. Eventualmente as alterações a nível de expressão fizeram-se sentir previamente, sendo agora mais presente uma actividade enzimática diferente entre uvas infectadas e

controlo. A enzima OMT1 assegura o crescimento e desenvolvimento das uvas assim como do seu aroma. Os resultados desta tese demonstraram existir o aumento do seu nível de expressão após a infeção nas uvas Trincadeira, mas não foi significativo.

No futuro, é fundamental voltar a repetir a análise aos voláteis e a estes genes, a partir de mais amostras (réplicas biológicas) e validar esta informação com outras estações produtivas para identificar marcadores metabólicos robustos. Outros genes/enzimas relacionados com o aroma e não estudados neste trabalho, também poderão ser analisados e assim complementar este perfil, inclusive através da análise de bagos menos maduros. Os fenóis e antocianinas foram quantificados para confirmar mais uma vez, as mudanças que surgem nas uvas devido à infeção pela *B. cinerea*. Para a determinação dos fenóis e antocianinas recorreu-se à liofilização das uvas e à sua quantificação por absorvância no espectrofotômetro após a sua extração por diferentes métodos.

Estes compostos são fundamentais para a produção do aroma e para a proteção da uva, através de uma barreira na sua pele criando inibidores de fungos que asseguram a qualidade das uvas e do vinho. Por norma, estes compostos defendem a uva contra o agente patogénico, mas, com uma grande infeção da *B. cinerea* nas uvas estas perdem a sua capacidade de defesa e ficando muito suscetíveis. Assim, verifica-se uma diminuição gradual destes compostos, no último estágio de desenvolvimento das uvas. Os resultados apresentados neste estudo demonstram essa mesma diminuição, facto confirmado em outras investigações.

Um estudo exploratório, para além do proposto inicialmente na tese, foi executado de modo a analisar os fenóis e antocianinas presentes nas uvas com e sem a hormona melatonina. Recentemente descobriu-se o papel desta hormona na proteção das plantas, contra danos, stress abiótico e biótico ou agentes patogénicos como o fungo *B. cinerea*. Ao tratar as uvas com melatonina promovemos a sua maturação e qualidade. Este estudo teve como objetivo determinar se a hormona adicionada exogenamente iria proporcionar alguma das características atrás referidas. Infelizmente, nos testes realizados nas uvas da Trincadeira e Touriga Nacional não se verificaram mudanças significativas ao nível da qauntificação dos fenóis e antocianinas que permitissem obter resultados pertinentes, sabendo-se ainda muito pouco acerca do papel da melatonina no último estágio de desenvolvimento das uvas.

Como conclusão, este trabalho permitiu identificar os compostos voláteis pertencentes ao aroma do sumo de uva da casta Trincadeira do ano 2018, com e sem infeção do fungo *B. cinerea*. A partir desta informação é possível identificar as mudanças que ocorrem no aroma da uva e determinar o perfil de compostos voláteis característicos da *Botrytis* cinerea na Trincadeira.

A expressão de alguns genes envolvidos no aroma sofreram alterações putativas envolvidas no aroma final das uvas da Trincadeira com e sem a presença do fungo. Estes dados englobam um conjunto de informação necessária para determinar e completar os dados anteriores referentes ao perfil dos compostos voláteis sobre o aroma das uvas com e sem a infeção da *Botrytis cinerea*. A determinação dos fenóis e antocianinas permitiu verificar mais uma vez a infeção e a gravidade que o fungo *Botrytis cinerea* produz nas vinhas.

Os resultados deste estudo poderão vir a ter impacto na decisão sobre a melhor forma de controlar ou minimizar a proliferação nas uvas, por parte deste agente patogénico. Poderá ainda vir a ser do interesse de alguns produtores vinícolas no que respeita às mudanças que ocorrem no aroma e qualidade das uvas após a infeção.

Palavras-chave: Trincadeira, *Botrytis cinerea*, compostos voláteis, compostos fenólicos, genes do aroma

Abstract

Grapes are one of the fruits more produced in Portugal. Worldwide they are very important economically.

Grapes from different grapevine varieties have their own characteristics, such as the colour and the aroma (mixture of certain concentrations of specific compounds). Trincadeira is one of the most important Portuguese grapes but is extremely susceptible to a fungal disease caused by *B. cinerea*. This fungus destroys partially or completely the grapes bunches, changing their quality and biological characteristics. By changing the quality of the grapes, their aroma also undergoes several modifications.

This study aims to characterize how Trincadeira grape aroma changes when infected with *B.cinerea*, through the analysis of free and glycosidic volatile compounds, the amount of phenols and anthocyanins and the expression of the genes (enzymes) responsible for volatiles production, during the last stage of development EL38 (harvest stage).

The volatiles were analysed using the Headspace solid-phase microextraction (HP-SPME) technique where the grape juice was heated in a water bath, the volatiles released and absorbed to be identified by gas chromatography and mass spectrometry (GC - MS). Initially, the free volatiles were determined and only after the addition of *β*-glycosidase enzyme the glycosylated volatiles were analysed. Based on the results of this study, several possible markers of infection were identified due to the presence of *Botrytis cinerea*. The analysis of volatiles showed the impact of *Botrytis* infection since some volatiles increased in response to the infection. During this analysis were found four groups of volatiles in grapes, the C_6 alcohols and aldehydes, alkanes, fatty acids and the amino acids derived esters and alcohols. The quantity of C_6 volatiles found in infected and non-infected grapes were similar. The alkanes found in the free form were lower in presence of *Botrytis*. The fatty acids volatiles appeared in the glycosidic form and increased during the infection. The amino acids derived esters and alcohols volatiles (free form) increased due to the infection. The glycosidic form of amino acids decreased during the infection.

Based on the volatiles identified in Trincadeira analysis and in the literature, were selected the various genes coding for enzymes involved in the synthesis of specific volatile compounds. The expression of the genes responsible for the aroma were analyse by real-time quantitative PCR (RT-PCR) to verify if they had a higher or lower level of expression, in response to the infection. The expression levels from RT-qPCR analysis of *HPL2, LOXC* and *OMT1* presented an increased during the infection. The *HPL2* is released when the grape is damaged or subjected to both abiotic and biotic stresses, it is also involved in resistance to pathogens. The *HPL2* had the highest expression levels and better statistical significance between non-infected and infected samples. *LOXC* is responsible for the production of aldehydes and alcohols and its expression level usually decreases in the harvest stage. However, during the infection its expression increases through not statistically significant. *HPL2* and *LOXC* are the genes responsible for the biosynthesis of C_6 aldehydes and alcohols volatiles, which also increased during the infection.

The GS21 enzyme is involved in amino acid metabolism and its function is to prevent the loss of aroma and the appearance of brown spots on the skin of grapes. In this study there isn't a conclusive result, since in both infected and non-infected grapes the expression levels were similar.

The enzyme OMT1 is involved in the growth, development and the aroma of grapes. The results show an increase of expression level during the infection in Trincadeira grapes, but it was not significant. These results indicate that changes in the transcriptional profiling of the genes involved in aroma probably occurred at an earlier stage of ripening.

Phenols and anthocyanins were quantified to confirm again the metabolomic changes in grapes due to *B. cinerea* infection. Phenols and anthocyanins were determined using lyophilized samples and quantified by

absorbance on the spectrophotometer after extraction by different methods. These compounds are essential in grape quality and for the protection of grapes through a barrier on grape skin creating fungal inhibitors that ensure the quality of grapes and wine. With *B. cinerea* infection grapes become very susceptible. The phenolic and anthocyanins content decreased during the infection by *Botrytis cinerea*.

An exploratory study, besides the one initially proposed in the thesis, was performed to analyse the phenols and anthocyanins present in grapes with and without melatonin hormone. The role of this hormone in plants is to protect them against damage, abiotic and biotic stresses such as the one cause by fungus *B. cinerea*. When grapes are treated with melatonin their ripeness and quality are promoted. This study aimed to determine if adding exogenously melatonin would provide any of the characteristics above. Unfortunately, in the tests carried out in Trincadeira and Touriga Nacional grapes, it were not found any significant changes in the phenolic and anthocyanin levels.

In conclusion, it was possible to identify the changes that occur in grape aroma and to determine the profile of volatile compounds characteristic of *Botrytis cinerea* in Trincadeira. The expression of some genes involved in the aroma underwent putative changes involved in the final aroma of Trincadeira grapes with and without the presence of the fungus. The determination of phenols and anthocyanins allowed to verify once more the infection and severity of *Botrytis cinerea* in the vineyards.

The results presented here, are a starting point for the understanding the aroma and quality changes of grapes when infected and could be vital for future vinicultural managements.

Keywords: Trincadeira, *Botrytis cinerea*, volatile compounds, phenolic compounds, aroma genes

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Abbreviation list

ADH	Alcohol dehydrogenase
AAT	Acetyltransferase
AH	Aldehyde isomerase
ABA	Abscisic acid
BADH	Betaine-aldehyde dehydrogenase
C_{13}	Six carbon
C_6	Thirteen carbon
CYP74B/C	Cytochrome P450 enzymes acting on the hydroperoxyl
CWDE	Cell wall degrading enzymes
cDNA	Complementary DNA
Cts	Cycle threshold values
DW	Dry weight
DEPC	Diethyl pyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
EL	Eichhorn-Lorenz
EDS1	Enhanced disease susceptibility 1
EtOH	Ethanol
FIDs	Flame ionization detectors
Fw	Forward
GLV	Green leaf volatiles
GS	Glutathione synthetase
GC	Gas chromatograph
GAE	Gallic acid equivalent
HPL	Hydroperoxide lyase
HS-SPME	Headspace solid phase microextraction
H_20	Water
IBMP	3-isobutyl-2-methoxypyrazine
IPMP	3-isopropyl-2-methoxypyrazine
IAA	Indole-3-acetic acid
ISA	Instituto Superior de Agronomia
JA	Jasmonic acid
LOX	Lipoxygenase
MPs	3-alkyl-2-methoxypyrazines
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NaCL	Sodium chloride
NaAc	Sodium acetate
OMT	*O*-methyltransferases
PAL	Phenylalanine ammonia-lyase

PAD4	Phytoalexin deficient 4
PDMS	Polydimethylsiloxane
PTFE	Polytetrafluoroethylene
KH_2PO_4	Phosphate buffer
KCl	Potassium chloride
RNA	Ribonucleic acid
Rev	Reverse
RT-qPCR	Quantitative Real-time Polymerase Chain Reaction
RPA	Peak area relative to the total peak area
SBMP	c3-sec-butyl-2-methoxypyrazine
SA	Salicylic acid
SD	Standard deviation
TPC	Total phenolic content
TFA	Trifluoroacetic acid
ΔΔCt	Delta delta cycle threshold values

1. Introduction

1.1. Grapevine varieties

Grapevine is one of the most common fruits growing in Portugal. They are well known for their sweet taste and juicy pulp. They grow during the summer and reach the maturation point around late August and early September. Grapes are very important due to their abundant applications. They can be consumed as a fresh or dried fruit or used to produce juice and wine (Perestrelo *et al.* 2018), granting numerous nutritional and health benefits for humans. They have also high value in some pharmaceutical, food and cosmetic industries due to their high content of antioxidants in form of polyphenols (Agudelo-Romero *et al*. 2015, Agudelo-Romero *et al*. 2013). It is important to define the characteristics of each grapevine variety since they are all different. Even when they belong to the same variety, growing in different locations can show different properties depending on the environmental conditions to which it is exposed. Also, in the same location, the same variety can show different characteristics during the years (Zarrouk *et al.* 2016).

Grapes can be separated into table grapes or wine grapes. Table grapes that are consumed freshly, are normally more attractive physically, for being larger, having a thinner skin, being seedless and with less acidity and sugar. While wine grapes are the total opposite of table grapes, the berries are smaller, with seeds and they are very sweet (Wu *et al*. 2016). *Vitis vinifera L*. grapes are a native family of grapes in Europe and they have different varieties such as red or white wine grapes. Some examples of red grapes varieties are Touriga Nacional, Trincadeira, Alicante Bouschet, Cabernet Sauvignon, Merlot, Syrah, Aragonez and Tempranillo; as white grapes there are Pinot Blanc, Chardonnay, Arinto, Fernão Pires, Moscatel, yellow muscat or Moscato Giallo but, these last two belong to the Muscat family of grapes (Fortes and Pais 2015).

Trincadeira is one of the most produced variety of red grapes in Portugal and used essentially, to make wine. Trincadeira is known to have excellent acidity, soft tannins and abundant intense aromas of plum and blackberry (Ali *et al.* 2011). It normally grows in a hot, dry and bright area with irregular yield. But, it´s extremely susceptible to fungal pathogens attacks such as grey mould caused by *B. cinerea*, which is one of the worst grape fungal diseases (Agudelo-Romero *et al*. 2015, Agudelo-Romero *et al.* 2014), causing quantitative and qualitative degradation in grapevines (Schueuermann *et al.* 2019). The *terroir* (soil) influence and change several characteristics of grapes and wines. For example, changing the produced compounds during the different development stages of grapes can formed a different aroma (Cabrita et al. 2007).

Touriga Nacional is an important native variety in Portugal. Unlike Trincadeira, Touriga has tiny berries with a high skin to pulp ratio, implying a higher amount of skin per berry that is related to the increased content in anthocyanins (Agudelo-Romero *et al*. 2013). The wine produced with Touriga Nacional is dark, full-bodied, powerful and very aromatic with rosemary and floral notes, similar to the scent of purple violet blossoms or blackberry (Ali *et al*. 2011).

1.2. Grape ripening

Grape berries present developmental stages acknowledged as the Modified Eichhorn-Lorenz (E-L) system (Fortes and Pais 2015). This development is characterised by a double sigmoid curve resulting from two consecutive stages of growth, separated by a lag phase as shown in figure 1.1. (Perestrelo *et al*. 2015, Agudelo-Romero *et al*. 2013).

Stage I: The first growth period (EL 31-34), named *preveraison* is the formation of seed embryos and the pericarp characterized by exponential cell division after fruit-set, followed by cell expansion before the berries enter the lag phase. Green berries accumulate organic acids, tartrate and malate and then start the biosynthesis of tannins (Coombe and Mccarthy 2000).

Stage II: Lag phase or *veraison* is the second development stage (EL 35), it is a transition phase characterized by a berry growth and softening with an expansion of volume to dilute the aroma precursors in juice precursors (Fang and Qian 2012) and initiate the change of colour development in grapes with accumulation of anthocyanins (Agudelo-Romero *et al*. 2013).

Stage III and stage IV: *Postveraison* stage (EL 36-38) or ripening is the last phase of berries development characterized by cell wall softening associated with an increase of pH, mainly due to the accumulation of soluble sugars, flavour-enhancing compounds, anthocyanins, fructose and glucose (Agudelo-Romero *et al.* 2013). The study of grapes volatiles compounds is normally done in this last stage (Coombe and Mccarthy 2000), where the berry volume is already increased with as high, and constant concentration of aroma precursors (Fang and Qian 2012).

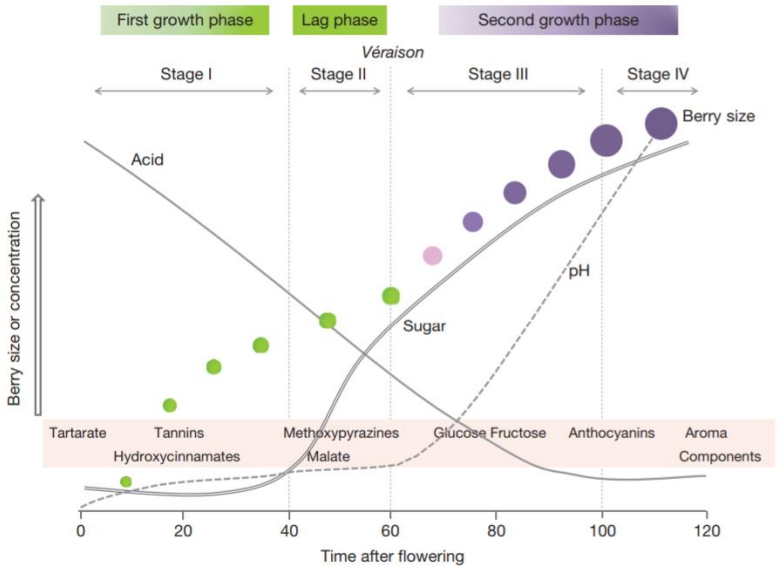

Figure 1.1. – Development stages of grape berries, showing color changes, size and compounds accumulation by Perestrelo et al. (2015). Where, stage I is the first growth period (EL 31-34) named preveraison, stage II is the lag phage or veraison (EL 35) and the last stage III and IV is characterized by postveraison stage (EL 36-38).

1.3. Primary and secondary grapes aroma

The aroma is one of the most important characteristics in fruit flavour, followed by the sense and taste. In grapes, the aroma is the accumulation of volatile compounds with high vapour pressures that can be released into the atmosphere crossing membranes freely from different plant secondary metabolites on different development berries stages (Agudelo-Romero *et al*. 2013). These volatiles compounds can be in a free form, contributing directly to the odour, or in a bound form, usually, as glycosides, producing the aroma potential (Fang and Qian 2012). Volatiles can be represented as aldehydes, esters, alcohols, terpenes, methoxypyrazines, ketones, lactones and other groups (Yang *et al.* 2009). Most cases show a high accumulation of bound form volatiles in grapes comparing to the free forms. The compounds present in grapes and wine are different, while in wine the compounds are in the bound form, grapes or juice have compounds in both forms (Cabaroglu, Canbas, and Gunata 2002, Gunata *et al*. 1985).

The aroma is divided in two classifications, the primary and the secondary aroma. The primary aroma gives information about the quality of grapes if they are aromatic or non-aromatic. For example, *Vitis vinifera* and Muscat are two aromatic grapes cultivars characterised by the many terpenes in both free and bound forms (Gunata *et al.* 1985). Trincadeira is a non-aromatic or neutral grape cultivar with aroma compounds lower than the respective odour thresholds present in free form. The content of bound volatiles in grapes can be similar to free volatiles of aromatic varieties (Lamorte *et al.* 2008), giving to non-aromatic grapes some aromatic potential (Genovese *et al.* 2013). Depending on the grapevine variety, the primary aroma can have different compounds such as C_6 aldehydes, C_6 alcohols, aromatic alcohols, terpenes, C_{13}-norisoprenoids and/or pyrazines, cinnamic esters, anthranilate esters and mercaptanes (Genovese *et al.* 2013). The secondary aroma is derived from thermal reactions, chemical enzymatic activity and fermentation processes (Cabrita et al. 2007). The glycosidic compounds are complex molecules usually volatiles esters, higher alcohols and aldehydes (Prudêncio Dutra *et al*. 2018).

1.4. Genes and volatiles compounds involved in grape aroma

During the grape development, the changes that happens in volatiles are very dependent on the enzyme activities. The grape aroma is formed through a mixture of some volatile compounds from different metabolomic pathways such as fatty acids, amino acids, alcohols, aldehydes, esters, terpenoid and others. Volatile compounds from fatty acids metabolomic pathways are derived from green leaf volatiles (GLVs) (Rambla *et al.* 2016). Green leaf volatiles (GLVs), are short-chain acyclic aldehydes, esters and alcohols. When something disturbs them, due to the presence of high lipid-derived six-carbon (C_6) aldehydes and alcohols compounds, they release a "green" aroma in berries, comparable to spice food and beverage industry aroma (Flagship and Osmond 2009). This "green" aroma can contribute to a bad aroma in wine. However, is also good when supplies substrates for the formation of esters during the fermentation processes (Lin, Massonnet, and Cantu 2019).

Aliphatic acid metabolism produces the GLVs through hydroperoxide lyases (HPLs) and alcohol dehydrogenase (ADH) catalysis. Damaging grapes or inducing biotic/abiotic stress, leads to membrane breakdown, releasing HPL and ADH. These two have an important role in helping grapes to recognizing insects or pathogens and to gain resistance to compete with them (OuYang *et al*. 2015). Rambla *et al.* (2016) reported that LOX-HPL pathway produce GLVs during grape development with the support of

lipoxygenases (LOX), ADH and HPLs activity.

Lipoxygenases (LOXs) are a family of fatty acid dioxygenases that target polyunsaturated fatty acids such as linoleic and α-linolenic acids, inducing the production of C_6 alcohols and aldehydes from free fatty acids (Lin *et al.* 2019). C_6 and C_9 aldehydes and oxoacids are also formed by hydroperoxides cleavage through hydroperoxide lyases (HPLs). In the end, through the lipoxygenase-hydroperoxide lyase (LOX-HPL) pathway, the C_6 compounds are direct products of oxidative cleavage of linoleic acid and α-linolenic acid (Rambla *et al*. 2016).

HPLs are CYP74B/C cytochrome P450 enzymes acting on the hydroperoxy functional group. They are separated into 9-HPL*s* (9(*S*)-hydroperoxides), 13-HPLs (13(*S*)-hydroperoxides) and 9/13-HPL*s* classes. Where HPL1 use 13(*S*)-hydroperoxides as substrate and HPL2 the 9(*S*)-hydroperoxides they produce hexanal, 2-*trans*-hexenal and *cis*-2-hexen-1-ol in *Vitis vinifera*. These volatiles compounds increase during ripening and their corresponding genes are normally low expressed until *veraison* and increase during maturation of berries.

The principal enzyme responsible for the biosynthesis of aldehydes and alcohols is lipoxygenase (LOX) and its expression levels decrease during the ripening stage (Van de Poel *et al.* 2014). LOX-HPL pathway is dependent on the activity of LOX, HPL, ADH, acetyltransferase (AAT) and aldehyde isomerase (AH). It has been reported that C_6 volatiles found during all the development stages in berries (*preveraison, veraison* and *postveraison*) increase when grape maturation starts. Free fatty acids compounds are toxic and catabolized by the lipoxygenase pathway consisting of the sequential enzyme activity of lipoxygenase (LOX) and hydroperoxide lyase (HPL) (Rambla *et al.* 2016). In the LOX pathway during ripening, the alcohol dehydrogenase (ADH) reduce the volatiles C_6 aldehydes produced to alcohols by the action of alcohol dehydrogenase (ADH) enzymes or by conjugation with glutathione (Lin *et al.* 2019). The conversion of ethanol into acetaldehyde during anaerobic fermentation induces the final aroma in grapes (Maoz *et al.* 2018). Aldehydes are produced from alcohols oxidation and released as aglycones and tannins (amino acids and alkaloids) present in the skin and seed tissues from grapes, being almost totally absent from the flesh (González-Barreiro *et al.* 2015). Studies show the expression of *ADH1*, *ADH2* and *ADH3* in berries with the accumulation of aliphatic alcohols. The expression of *ADH1* and *ADH3* increase until *veraison* and then decrease. While the *ADH2* presents low expression before *veraison*, increasing the expression and enzyme activity later, being the most express gene of the other two *ADHs*. ADH releases alcohols to produce the grapes and wine flavour or to act as substrates producing esters by yeast alcohol acyltransferases (AAT) (Lin *et al.* 2019). AAT use substrates from the lipoxygenase pathway and amino acid metabolism to produce hexanoate esters (Lin *et al*. 2019).

Volatile esters and acetates are derived from amino acids and essential fatty acids metabolism with aldehydes as intermediate produced by decarboxylation and deamination reactions. Once more, these volatiles increase since *veraison* until ripening. Afterwards, the aldehydes are reduced to the respective alcohols followed by acylation (acetyl-CoA) reactions and catalysed by enzymes from the BADH gene family such as AAT (Vilanova *et al.* 2013). This reaction includes amino acids such as 2-phenyl ethanol and fatty acids derived from C_6 alcohols (hexanol for example). Generally, volatile esters in grapes appear with low concentration and emerge in grapes due to yeast metabolism during the fermentation process

(Maoz *et al*. 2018). Esters due to yeast metabolism through fermentation processes are found in bigger quantity producing a good wine aroma with fruity notes (Vilanova *et al*. 2013). Although normally grape berries don´t accumulate many volatile esters, only a few such as acetates of short-chain alcohols still have some biosynthetic potential to convert exogenous amino acids into volatile compounds.

Glutathione synthetases (GSs) are the enzymes involved in amino acid metabolism (Rambla *et al*. 2016). One of the GS functions is to prevent aroma loss and reducing the skin browning in grapes due to the oxidative decay from the dissolved oxygen in wine (Zacharis *et al.* 2013). The GSs enzymes are synthesized through the phenylpropanoid pathway, where phenylalanine ammonia-lyase (PAL) produce (E)-cinnamic acid with the support of *O*-methyltransferases (OMTs) (Rambla *et al*. 2016). The phenylpropanoid pathway is one of the most important secondary metabolism pathways, controlled by *PAL* genes responsible to the anthocyanin biosynthesis (Villegas *et al.* 2016), producing some metabolites, such as benzoic acids, stilbenes, anthocyanins and flavonoids, crucial to the fruit development, maturity, quality and attractiveness (Fock-Bastide *et al*. 2014). Volatiles from amino acids are derived from phenolic compounds or branched-chain compounds (Rambla *et al.* 2016).

MPs (3-alkyl-2-methoxypyrazines) in plants are nitrogen-containing heterocyclic compounds, biosynthesized (Lei *et al*. 2019). MPs are also called flavourants for being very important in red wine grapes producing a herbaceous, green bell pepper aroma compounds at harvest stage (Xi *et al*. 2016). This aroma is created due to three MPs compounds, the 3-isobutyl-2-methoxypyrazine (IBMP), for the green pepper aroma in some grape varieties, the 3-isopropyl-2-methoxypyrazine (IPMP) rarely detected in grapes and wines and 3-sec-butyl-2-methoxypyrazine (SBMP), rarely detected (Lei *et al*. 2019). MPs are mainly located in berry skins and its pathway initiates with the condensation of NH_3 with an amino acid to form 3-alkyl-2-(1H)-pyrazin-2-one and 3-alkyl-2-hydroxypyrazine (HPs). During MPs biosynthesis, the HPs are *o*-methylated to form MP. The *O-methyltransferase (OMT)* genes found involved in *V. vinifera* are the *OMT1, OMT2, OMT3,* and *OMT4* (Xi *et al.* 2016). The accumulation of MPs was associated with the expression of *OMT1* in the skin and flesh of developing Cabernet Sauvignon grape berries, and the higher *OMT2* expression in roots (Xi *et al.* 2016). OMT is a structural enzyme responsible for methylation downstream of anthocyanin glycosylation (Villegas *et al*. 2016). *O*-methyltransferases 2 and 3 (*OMT2* and *OMT3*) can be involved in the biosynthesis of phenolic and flavonol components (Fock-Bastide *et al.* 2014). *O*-methyltransferases (OMTs) can methylate substrates such as phenolic compounds, changing its structural polarity (Ju *et al.* 2019), caffeoyl-CoA esters, hydroxycinnamic acids, simple phenols, carboxylic acids, (iso)flavonoids, and alkaloids. Some important secondary metabolites to support the growth and development of plants are catalysed by the *O*-methyltransferases (OMTs). For example, phytoalexin protects plants from phytopathogens infection or stresses; an important aromatic compound of phenylpropenes contributes to flavour and aroma; lignin is important for cell wall formation, small molecules transportation and resistance of pathogens like fungus (Z. Liu *et al.* 2018).

Volatiles terpenoids normally derived from aromatic grapes are responsible for the floral notes in grapes (Vilanova *et al.* 2013). Terpenoids in grapes are involved in the biological activity, defence, primary metabolism, aroma production (Martin *et al*. 2010), anticarcinogenic, antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory and antiparasitic activities (Perestrelo *et al.* 2018).

Trincadeira grapes are neutral or not aromatic, and because of that, it doesn´t present terpenoids volatile

compounds. One consequence of being neutral is the loss of capability to have antifungal activity due to the absence of terpenoids volatiles, turning this variety more susceptible to pathogens attacks such as *B. cinerea*.

1.5. HS-SPME combined with GC and GC-MS

The main method used for quantitative analysis of complex mixtures (Zhao *et al.* 2019) to determine the volatile compounds in juice or wine is the gas chromatography coupled to mass spectrometry (GC-MS) (Prudêncio Dutra *et al.* 2018). GC-MS is a good technique to identify unknown compounds comparing target fragments using matched mass spectra in the current libraries and the corresponding compounds from standard references. The advantage of this method is the separation of each compound obtained based on their retention time (retention indices) (Majchrzak *et al*. 2018). Sometimes, the concentrations of some volatile compounds with high odour intensities are too low to be detected by GC–MS (Song and Liu 2018).

The other method is the headspace solid-phase microextraction (HS-SPME), which requires a low volume sample, no organic solvents, the analysis is less expensive, less time-consuming and easily automatable when compared to methods using solvent extraction, allowing the extraction and pre-concentration of analysis simultaneously (Moreira et al. 2019). SPME, first proposed by Pawliszyn and Arthur (1990) is a simple, solvent-free, extraction technique for organic compounds in aqueous samples. In this process the analytes are adsorbed directly from the samples onto a fused silica fiber that is coated with an appropriate stationary phase. The analytes partition from the sample matrix into the stationary phase until equilibrium is reached when the fiber is inserted in the sample. And then, the fiber is inserted into the injector port of a gas chromatograph (GC) and mass spectrometry (GC–MS) where it is heated, and the analytes are rapidly thermally desorbed into a capillary GC column for analysis. The main disadvantages of SPME are the limited sorption capacity and the displacement effect of analytes with a lower affinity for the coating (Majchrzak *et al*. 2018). When we have an appropriate set of analytical techniques like SPME together with GC-MS, we get a good sensitivity of the analytical method allowing the identification and further determination of chemical compounds (Majchrzak *et al.* 2018). SPME give us the appropriate sample preparation technique with the adsorption of volatiles and their isolation in GC-MS to determination of grape aroma.

1.6. Infection of grape by *Botrytis cinerea*

All plants, during their lifetime, are exposed to a wide range of pathogens and become susceptible to them, such as viruses, fungi, bacteria and nematodes. Grapevines are vulnerable to diverse pathogens as shown in figure 1.2. Some of these pathogens are necrotrophic (i.e., *Botrytis cinerea*), biotrophic and hemibiotrophic (i.e., *Erysiphe necator, Plasmopara viticola, Agrobacterium vitis, Xylella fastidiosa*) and viruses (≈70 virus species) (Armijo *et al*. 2016).

Botrytis cinerea is a fungus that infects not only grapevines but more than 200 different plant species in the world (Teissedre 2012). *B. cinerea* works in grapevines as a parasite and is an ubiquitous, filamentous and typical necrotrophic fungus that lives in green tissues or saprophyte inside dead tissues, contributing to his spreading in nature and host unspecificity (Armijo *et al.* 2016). *Botrytis cinerea* attacks during flowering (bloom), after *veraison* (Teissedre 2012) and sometimes through rainfalls, periods with high humidity and

when grapes have some sensitivity in the post-harvest stage, destroying partly or entirely the berry clusters (Armijo *et al.* 2016).

There are two different ways of infection in grapes by *B. cinerea*. The first is due to the formation of skin pores or by mycelium penetration through wounds. The second happens through conidia infection followed by germination in the flower receptacle (host) and latency until berry maturation (Viret *et al*. 2007).
Botrytis cinerea conidial (C) is stimulated and germinated through exudates from mature fruits dependent on the presence of sugar and other nutrients, producing the primary lesions (Cotoras, García, and Mendoza 2009). The primary lesions called appressorium structures, activates an oxidative burst that helps the fungus to kill, degrade, germinate and penetrate the grape berries tissues by necrosis crossing the cuticle and outer epithelial wall (i.e. cutinases and lipases). This necrosis arise through the secretion of toxic compounds or lytic enzymes and cell wall degrading enzymes (CWDE), such as endopolygalacturonases, pectin methylesterases, cellulases and hemicellulases (Armijo *et al.* 2016). When these layers suffers a break or penetration, occurs a decomposition and consumption of the plant biomass (Armijo *et al.* 2016) and the reduction of host defences by killing the cells host (Jan 2006), causing the grey mould disease shown in figure 1.2.

Nowadays many researchers want to understand how *Botrytis* adapts to various environmental conditions, destroying harvest stage berries and to know how the biochemical, biology and genetic basis of *B. cinerea* resistant against fungicides. *B. cinerea* infects grapevines causing economical and oenological qualitative consequences (Teissedre 2012). Concerning the quantitative aspects, the yield is reduced at harvest. When we talk about oenological quality impact, some changes occur in grape berry chemical composition and some qualitative compounds such as sugars, organic acids, varietal aromas and phenolic compounds (Teissedre 2012, Bocquet F MD 1995, Agudelo-Romero *et al*. 2015). Nevertheless *Botrytis cinerea* may be beneficial under certain climatic conditions in grape berries, to produce sweet wines with prices up to €500/bottle (Dean *et al.* 2012). What normally agricultures do is through chemical control spraying aerial parts of vines (fructiferous zone) with specific anti-*Botrytis* fungicides, reducing the incidence and severity of this fungus. However, exists a negative perception by the public regarding the safety of pesticides (Gullino and Kuijpers 1994), the concerning of change in chemical, morphological and anatomical characteristics of grapevines (Gabler et al. 2007), impeded by the development of resistant strains to many botryticides (Elad and Evensen 1995).

Many countries and people want to keep increasing the green environmental policies concerning the use of chemicals in farming zones and for that, new techniques such as biofungicides, mineral oil and plant hormones need to be found (Jacometti, Wratten, and Walter 2010). Nevertheless, until effective resolutions against *B. cinerea*, it will continue to be a concern for producers of grapes and wines.

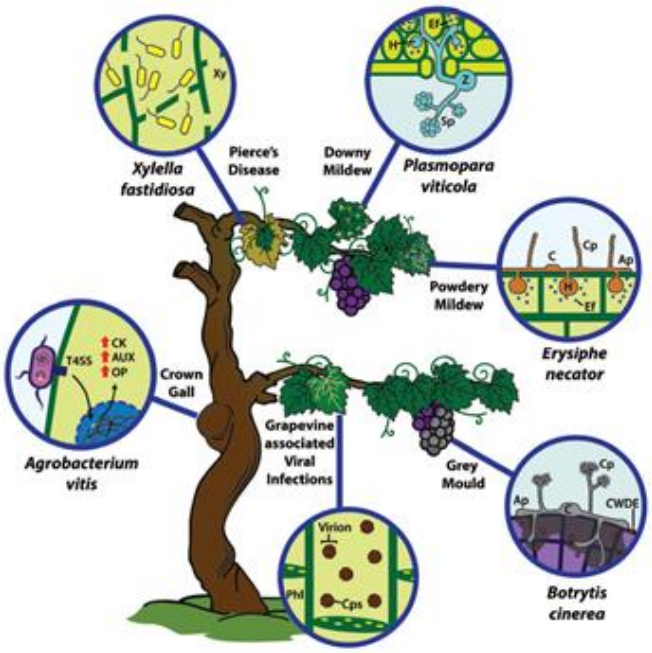

Figure 1.2. – Scheme of infection strategy and disease-associated symptoms of main grapevine pathogenic microorganisms (Image from Armijo *et al*. 2016). Infection by necrotrophic (i.e., *Botrytis cinerea*), biotrophic and hemibiotrophic (i.e., *Erysiphe necator*, *Plasmopara viticola*, *Agrobacterium vitis*, *Xylella fastidiosa*) and viruses pathogens disease.

1.7. Primary and secondary metabolism and hormones during grape infection and defense

Grape quality is determined through the primary and secondary metabolomic profile. Primary metabolites (i.e. proteins, lipids, nucleic acids) are found in all grapevines and give them the daily functions and biological processes. On the other hand, secondary metabolites are known to have physiological functions such as adaptation to the environment, acquired resistance to pests and diseases, building of symbiotic relationships with microorganisms and pollinator attractant capacity (Ali *et al*. 2010), responsible for the chemical diversity of grapevine, and have many phytochemical groups (such as alkaloids, terpenoids, tannins, sterols, saponins, and phenolics). Most of these secondary metabolites are valuable for the pharmaceutical, agrochemical, food, and fragrance industries (Perestrelo *et al.* 2018).

The biosynthesis from secondary metabolites is different in every grape cultivar and gives resistance against several pathogens. When grapevines are being attacked by a pathogen the skin create a spectrum of many defence responses with the synthesis of many compounds (i.e., anthocyanins and aroma volatiles) (González-Barreiro *et al.* 2015) and, the development of a mechanical protective barrier in the skin and epidermic cell layers, creating fungal inhibitors from skin with phenolic compounds (Teissedre 2012). Phenolic compounds are a class of secondary metabolites very important to the quality of grapes and wine. The main classes of phenolic compounds are stilbenes, anthocyanins and proanthocyanidins (condensed tannins) (Teissedre 2012). Anthocyanins and proanthocyanidins came from seeds and skin of grapes, and they are very important to give berries color through chemical reactions with anthocyanins by copigmentation and condensation (Teissedre 2012). Anthocyanins are located principally in red grape skins (González-Barreiro *et al.* 2015) appearing after *veraison* in the second grown period (Tassoni *et al.* 2019) creating the pigmented compounds responsible for red wine colour as shown in figure 1.1. (Teissedre 2012).

Teissedre (2012) describe that proanthocyanidins can maintain *B. cinerea* in a quiescent stage, leading to late development of symptoms and act as competitive inhibitors against *B. cinerea* (Kunz *et al*. 1999). The phenolic compounds have an important role during the defence against *B. cinerea* and give vital properties to juice and wine from an oenological point of view (Teissedre 2012). Normally with the infection there´s a gradual decline of phenolics compounds and the amino acids and sugars are being accumulated in berries during the later stages of development (Ali *et al.* 2011). Previous NMR studies proved that Trincadeira has high sugars, organic acids and low phenolics content. However, Touriga Nacional has high phenolics content and specific amino acids (Agudelo-Romero *et al.* 2013).

Some secondary metabolites, a complex interplay among different hormones (Coelho *et al.* 2019) and expression of specific genes (Agudelo-Romero *et al.* 2015) promote a certain resistance and defence against many pathogens and diseases in grapes. Grape berries have resistance against different infections during bloom and *veraison*, due to natural and pre-existing skin defence structures (such as cell layers, cuticle and wax content of the fruit, stomata and leaf trichomes) (Armijo *et al.* 2016) and induce the biosynthesis of defensive compounds, such as alkaloids, phenolic compounds, flavonoids and terpenoids (Kunz *et al.* 1999). The deterioration of phenolic compounds is due to fungal oxidasic and esterase activities with the conversion of terpenes into less odorous compounds and also, for hydrolysis of fatty acids increasing their biosynthesis (Barata, Malfeito-Ferreira, and Loureiro 2012, Tassoni *et al.* 2019). Pathogens like *B. cinerea* can bypass this interplay and inactivate specific genes necessary for plant defence and resistance. Previous studies revealed that hexokinase activity is required for *B. cinerea* development, being able to manipulate the metabolism to use carbon sources for its growth with products of tissues degradation (Armijo *et al.* 2016).

Hormones are crucial to the development and ripening of grapes, ensuring the regulation of grapes development through all growth stages. Abscisic acid (ABA), brassinosteroids (BRs) and ethylene hormones promote the ripening. ABA is known to influence the accumulation of sugars, producing of berry softening and skin coloration, induce or repress plant defense and is determined by their biosynthesis, conjugation and catabolism. Using exogenous ABA before veraison can increase the anthocyanin content and the biosynthesis of phenylpropanoids. Auxins on the other hand, has an important role in fruit growth, but act as inhibitors of ripening, berry size, sugar accumulation, and anthocyanin content. They regulate negatively ABA-induced ripening processes. Indole-3-acetic acid (IAA) belongs to the auxin class, and its concentration increase during the early development stages of grapes and in final stages decreases (Coelho *et al*. 2019). Plants hormones like salicylic acid (SA) and jasmonic acid (JA) have a role in the defence response against pathogens and they are positive regulators of plant defense. JA activates an immune response against this pathogen (Coelho *et al*. 2019). SA decreased during ripening in Trincadeira grapes. Involved in SA signalling are the *enhanced disease susceptibility 1* (*EDS1)* and *phytoalexin deficient 4 (PAD4)* genes with the role of disease resistance (Agudelo-Romero *et al.* 2015). Jasmonic acid content influences the colour and aroma of grapes. A volatile form of JA, methyl jasmonate when applied exogenously in grapes, induces the red colour, increasing the anthocyanin content and the volatiles´ synthesis (Coelho *et al.* 2019). JA and auxins decreased during ripening and ABA increase at *veraison* in Trincadeira and decreased again at harvest. It has been reported that ABA is associated to disease resistance but ABA can suppress plant resistance mechanisms (Coelho *et al*. 2019). Besides, with the infection some pathogens can produce ABA accelerating the ripening in the host increasing their susceptibility to them (Coelho *et al.* 2019). Grapevines are more susceptible in the last stages with mature berries than in

veraison. In *postveraison* berries occur the activation of the jasmonic pathway, being unable to stop the fungal infection (Rascle *et al*. 2015). However, they can harbour the pathogen as endophytic without any visible signs of disease development.

Melatonin is a hormone found in almost every living beings, animals, plants and microbes (Meng *et al.* 2018). Some reports suggest that melatonin can protect plants against damage caused by various biotic and abiotic stresses like the presence of fungus *B.cinerea* (Meng *et al*. 2015). Besides its function as synchronizer of the biological clock, it is also a powerful free-radical scavenger and wide-spectrum antioxidant (C. Liu *et al*. 2018) and plays regulatory roles in plant metabolism, acts as a growth-regulatory signal similar to auxin (Lili *et al*. 2018), delays flower induction, slows root formation, and promotes adventitious and lateral root regeneration. A recent investigation by Meng *et al.* (2018) identified melatonin in all grape berry tissues (skin, flesh, and seed). During *preveraison*, melatonin content was higher in berry skin, at *veraison* in the seeds and at ripening, occurs a decline in the skin and an increase in the seeds and flesh (Meng *et al.* 2018). Some researchers tried to use exogenous melatonin as an effective treatment in plants to promote ripening and improve quality of fruits (C. Liu *et al.* 2018). However, the role of melatonin during berry ripening is still unclear (Sun *et al.* 2015, Lili *et al*. 2018). The perception of different stress hormones will give information about the plant resistance against pathogens (Coelho *et al.* 2019).

1.8. Scope and objectives of the study

This study has the objective to identify the volatile compounds, determine the phenolic and anthocyanins content and the expression of genes involved in the formation of Trincadeira grapes aroma when occurs an infection with the fungus *Botrytis cinerea*. Ultimately, this thesis aims to understand how these changes can affect the quality of grape aroma and wine during the ripening stage (EL 38).

Trincadeira is a common variety used in the production of good wines, and because of that some studies have been conducted to analyse its aroma compounds. However, we still need to know more about the changes in aroma due to the infection with *B.cinerea* in Trincadeira that will lead to aroma modifications, producing at the end undesirable volatile compounds. This work will allow to analyse the difference between aroma profiles of control and infected berries (free volatile and the bound volatiles). Additionally, the expression of genes involved in aroma development will also be evaluated, together with the analysis of phenolic and anthocyanins content.

In the end, the study of genes involved in Trincadeira aroma will provide information in order to understand which genes and metabolic pathways and volatile compounds formed are more subjected to change due to *B. cinerea* attacks. Furthermore, this kind of information is useful to comprehend how *B. cinerea* attacks grapes and changes aroma quality of grapes and wine. In the future, this study can support other investigations to discover a better method to prevent the infection and help the grape and wine producers to monitor the degree of infections.

2. Experimental Methodology

2.1. Fungal infection of berries and sample collection

Infections of grapevine berries with *Botrytis cinerea* were made in June 2018 at the Instituto Superior de Agronomia (ISA), University of Lisbon, Portugal. The isolation procedure of *B. cinerea* was obtained from diseased grapevine plants and maintained in potato dextrose agar (Difco, Detroit, MI, USA), at 5 °C. Afterwards, the inoculated Petri dishes with potato dextrose agar were exposed to continuous fluorescent light, at 24 °C, producing the conidia. Conidia were harvested from cultures and collected by rubbing with phosphate buffer (0.03 M KH_2PO_4), filtered through cheesecloth to remove mycelia, and the concentration adjusted to 10^5 conidia mL^{-1}. The infections were made by spraying berry clusters with a conidial suspension at the developmental stage of peppercorn size (stage EL29), following the procedure by Agudelo-Romero *et al.* (2015) describe above. Collection of Trincadeira samples was performed at harvest (stage EL38) stage of development (Appendices, Figure 1). The bunches of non-infected (control) and infected grapes were obtained, and all transported briefly to the laboratory on ice. One part of the bunches was for analysis of the volatiles and another one immediately frozen in liquid nitrogen and stored at -80 °C until further use.

2.2. Determination of free and glycoside bound volatiles

2.2.1. Volatiles isolation protocol optimizations

Preliminary Headspace solid phase microextraction (HP-SPME) experiments were run using different parameters to assess the most adequate SPME conditions. Assessed parameters evaluated were the volume of grape juice (3, 5, 7 and 15 mL), the addition, or not of NaCL (0.2 g NaCL/mL), and for glycosidic bound volatiles, the amount of *β*-glycosidase added (10, 12 and 15 mg/mL). The conditions described below were those finally adopted.

2.2.2. Volatiles isolation procedure

Free volatiles were isolated from the juice of freshly harvest Trincadeira grape bunches, from non-infected (control) and infected grapes (Appendices, Table 1) The grapes juice was obtained by maceration from approximately 100 g of grapes inside a beaker with a strainer and a pestle at room temperature. To each grape juice macerated, 0.2 g NaCl/mL were added according to Agudelo-Romero, Erban, *et al.* (2013). To isolate the volatiles from the grapes juice, Headspace solid phase micro extraction (HP-SPME) assays were performed using a 100 μM polydimethylsiloxane (PDMS) coated fibers (Supelco, USA) inserted into a manually operated SPME holders. Each SPME fiber was thermally conditioned for up to 30 min at 250 ºC before use, according to the manufacturer recommendations. The grapes juice was inserted into two 22 mL clear vials, closed with a screw top solid cap with polytetrafluoroethylene (PTFE) liner, accommodated in a support rack inserted into a Bains Marie bath (Univeba) for gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) analysis. The fibers were exposed in each vial, for 30 min at 50 ºC for analysis of free volatiles. Blank experiments of the fibers and of empty vials were carried out regularly. The volume inside each vial, the code used to every SPME fiber and the date from every analysis was register in Appendices, Table 2.

2.2.3. Determination of glycosidic bound volatiles

After the isolation of free volatiles, the remaining grapes juice was evaluated for glycosidic bound volatiles. The isolation of glycosidic bound volatiles was made according to the Baerheim Svendsen and Merkx (1989) method, where around 100g of each bunch of from non-infected (control) and infected grapes (Appendices, Table 1). were macerated the same way as before. The hydrolysis was performed adding 10 mg/L of *β*-glycosidase enzyme from almonds (lyophilized powder, ≥ 2 units/mg solid–Sigma-Aldrich®) to the juice and then mix and homogenized with the enzyme at room temperature. The 22 mL clear vials stayed overnight in a water bath for 24 h at 37 °C inside Orbisafe TS Nwt Wise incubator with 15 rpm stirring to stimulate the emission of the glycosylated volatiles. The volatiles obtained after hydrolysis were isolated the same way as free volatile described above with an exception of fibers time inserted in each vial for 1 h at 50 ºC. The volume inside each vial, the code used to every SPME fiber and the date from every analysis was register in the Appendices, Table 2.

2.2.4. Volatiles analysis

All isolated volatiles, that is, volatiles from the juice of non-infected (control) and infected grapes, and glycosidic bound volatiles, were analysed by gas chromatography-mass spectrometry (GC-MS) for component identification, and by gas chromatography (GC) for component quantification.

2.2.4.1. Gas chromatography (GC)

Immediately after sampling, the SPME needle was introduced into the injector of a PerkinElmer Clarus 400 gas chromatograph equipped with two flame ionization detectors (FIDs), a data-handling system, and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (100% polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column [(50 % phenyl)-methylpolysiloxane, 30 m x 0.25 mm i.d., film thickness 0.15 µm; J & W Scientific Inc.]. Analytes desorption was achieved in splitless mode at 250 °C for 1 min. Oven temperature was programmed for 45–175 °C, at 3 °C/min, subsequently at 15 °C/min up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures were 250 °C and 300 °C, respectively; and the carrier gas (hydrogen) was adjusted to a linear velocity of 30 cm/s. Analytes desorption was confirmed by subjecting the SPME fiber to a second run, which always showed no carry-over peaks.

2.2.4.2. Gas chromatography–mass spectrometry (GC–MS)

Directly after sampling, the SPME needle was introduced into the split/splitless injector of the GC–MS unit that consisted of a PerkinElmer Clarus 600 gas chromatograph, equipped with DB-1 fused-silica column (100% polydimethylsiloxane, 30 m x 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.), and interfaced with a Clarus 600T Mass Spectrometer (software version 5.4.2.1617, Perkin Elmer, Shelton, CT, USA). Injector and oven temperatures were as above; transfer line temperature, 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; splitless mode; ionization energy, 70 eV; scan range, 40–300; scan time, 1s. The identities of the components were assigned by comparing their retention indices, relative to *n*-alkane indices and GC–MS spectra from a lab-made library, which was constructed based on reference essential components confirmed by RI, GC–MS and by 13C-NMR, laboratory synthesized components and commercially available standards (Extrasynthese, Fluka,

Riedel-de Haën and Sigma-Aldrich).

2.3. Determination of total phenolic content

The total phenolic content (TPC) was determined by spectrophotometry, using gallic acid as a standard, according the method described by Singleton and Rossi, (1965) with slight modifications. The grapes were freeze-dried for 72-96 h at 40 °C. The samples were weighed (Appendices, Table 4), and approximately 25 mg transferred to Eppendorf tubes and 0.2 mL of water was added. The same procedure was carried out for methanol to try a different extraction solvent. The tubes were centrifuged at 12.900 rpm for 40 minutes. Briefly, 0.2 mL of the diluted sample extract was transferred to tubes containing 1.0 cc of a 1/10 dilution of Folin-Ciocalteu's reagent in water. After waiting for 10 minutes, 0.8 mL of a sodium carbonate solution (7.5% w/v) was added to the sample. The tubes were then allowed to stand at room temperature for 30 min before absorbance at 743 nm (water) and 765 nm (methanol) was measured. The TPC was expressed as mg/mL of gallic acid equivalents (GAE) per mg of lyophilized grapes. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 0.0125 to 0.1 mg/mL (0.0125, 0.025, 0.05 and 0.1 mg/mL).

2.4. Determination of total anthocyanin content

Anthocyanin concentration was measured as described previously by Coelho *et al.* (2019). Grapes at -80°C were frozen in liquid nitrogen, the seeds removed, and grapes macerated. Grapes were freeze-dried for 72-96 h at 40 °C and then approximately 50 mg (Appendices, Table 6) of the powder was extracted in 1.5 mL TFA (Trifluoroacetic acid)/methanol/H2O (0.05/80/20, v/v/v). Samples were vortexed for 1 min and then anthocyanins were extracted for 1 h on ice in Eppendorf tubes. The mixture was then centrifuged for 30 min at 13,000 rpm at 4 °C. A 100 μL of this sample was diluted to 1 mL in extraction solution for *veraison* and harvest samples. The solution was mixed and allowed to sit for 5 min before reading the absorbance at A520. Total relative anthocyanin concentration was expressed as the absorbance value at 520 nm g^{-1} of freeze-dried weight (DW).

2.5. Gene expression analysis

2.5.1. RNA extraction from grape

RNA extraction was carried out according to Fortes *et al.* (2011). with slight modifications. Grapes were macerated and grounded to a fine powder in the liquid nitrogen using the mortar and pestle after removing the seeds. Grapes macerated were placed in a tube (falcon 50 mL) until 6.5/7.5 mL. This transference was made with liquid nitrogen to ascertain that the grapes didn´t unfreeze and the RNA didn´t degrade. The extraction was done in RNA buffer (Tris-HCl 1M pH 9, 1% SDS, 0.8% PVP-40, 5% *β*-mercaptoethanol). Added up to 16.5 mL, mixed and put on ice. The mixture of 1 vol. Chloroform:isoamyl (24:1) was added to the falcon and vortex 5 seconds and centrifuged at 6900 rpm, 25 minutes, at 4 °C. The aqueous layer obtained after centrifugation was transferred to a new tube and was performed a treatment with 2M KCl, 0.16M final concentration. Samples were mixed gently by inversion and kept 50 minutes in ice to precipitate the proteins. The mixture was centrifuged again at 6900 rpm, 40 minutes, at 4 °C to remove any remaining insoluble material and transfer the supernatant to previously sterilized Corex tubes for RNase free. To the supernatant, 1/10 vol. of sodium acetate (NaAc) 3M, pH 5.2 and 0.8 vol. of isopropanol were added to

precipitate nucleic acids, mixed thoroughly with parafilm and centrifuged at 6900 rpm, 30 minutes, at 4 °C and discarded the supernatant. Washed (5 mL) with ice-cold 70% EtOH-H_20 DEPC, centrifuged at 6900 rpm, 15 minutes, 4°C after each and discarded the supernatant and dried in *hotte* for 15 minutes. The pellet was solubilized in 1.4 mL of DEPC water, in 2 mL Eppendorf tubes. And treatment of polysaccharides with 1/10 vol KAc 2M and 45 minutes on ice was performed followed by centrifugation at 6900 rpm, 30 minutes at 4 °C. The supernatant was transferred to two 1.5 mL separate Eppendorfs. In order to selectively precipitate RNA, 1 vol. of 4M lithium chloride (LiCl) was added and the sample was kept overnight on ice.

The following day, the RNA was obtained in the form of pellet by centrifugation at 13000 rpm for 20 minutes at 4 °C and then washed twice with 500 µL ice-cold 70% EtOH-H_20 DEPC, vortex and then centrifuged at 13000 rpm, 8 minutes at 4°C. Finally, RNA pellet was air dried for 30 minutes inside the *hotte* and dissolved in 100 µL of DEPC-treated water and stored at -20ºC. The purity of RNA was confirmed by the absorbance ratio of 260/280nm to quantify the proteins (~2.0) and 260/230nm to quantify the other contaminants (polysaccharides) (~2.3) from 1 µL of each RNA sample using the Nanodrop ND-1000 spectrophotometer.

2.5.2. RNA purification and quality assessment

RNA was purified using a DNase treatment, performed according to the supplier's instructions (Invitrogen, San Diego, CA, USA) with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). To the final Eppendorf tubes from RNA extraction performed before, 300 µL of binding solution were added, after which the samples were mixed with a pipette. The mixture was transferred to a binding column in a 2 mL collection tube, which was centrifuged at 13500 rpm for 1 minute. To the mixture was added 300 µL of wash solution 1 and centrifuged at 13500 rpm for 1 minute. DNase digestion was performed for which the DNase I stock solution was prepared. By adding to the binding column 80 µL of the mixture made by mixing 10 µL DNase I, 70 µL DNase digestion buffer and 0.5 µL ribolock, and allowed to stand at room temperature for 15 minutes. After this, 500 µL of wash solution 1 was added and centrifuged ate 13500 rpm in 1 minute. Two more identical centrifugations (30 seconds) were executed after adding 500 µL of wash solution 2. After each centrifugation, the binding column was removed, and the collector tube was dried. The column was then dried by centrifuging during 1 minute at 13500 rpm, after which it was put in a new collector tube. To the centre of the membrane was added carefully 40 µL of elution solution, followed by 1 minute at room temperature and a short centrifugation at 13500 rpm, 1 minute to elute the sample. The purity of RNA was again confirmed by the absorbance ratio of 260/280nm to quantify the proteins (~2.0) and 260/230nm to quantify the other contaminants (~2.3) from 1 µL of each RNA sample using the Nanodrop ND-1000 spectrophotometer. After the purification step of RNA, a 1,1% agarose gel in 1 x TAE buffer was used to verify the quality/integration of the RNA. The agarose gel contained 3 µL of GelRed® nucleic acid gel stain.

2.5.3. Reverse transcriptase protocol

To generate first-strand cDNA, 2 µg of total RNA after were used and the Thermo Scientific RevertAid H Minus Reverse Transcriptase protocol was followed. For each sample, 11,5 µL of RNA with DEPC water and 1 µL of oligo-dT primers were added to a sterile Eppendorf tube. The tubes were mixed gently and briefly spin-down before incubating at 65°C for 5 minutes, after which they were put on ice and briefly spin-down a second time. To each tube was added 4 µL of reaction buffer (5X), as well as 0,5 µL (20 U) of

Thermo Scientific™ RiboLock™ RNase Inhibitor, 2 µL of a dNTP mix and 1 µL (200 U) of RevertAid H Minus reverse 13 transcriptase, in a total volume of 20 µL. The tubes were mixed gently and spin-down, (briefly centrifuged at maximum speed) and incubated at 42°C for 60 minutes, after which they were stored at -20°C.

2.5.4. Primer selection

For qPCR, primers involved in aroma development were selected based on previous SPME GC-MS volatile analysis and analysis of volatile metabolism and bibliography from Rambla *et al.* (2016). The primers selected are presented in table 2.1.

Table 2.1.- Oligonucleotide sequences (primers) used for gene amplification in RT-qPCR reactions.

Gene (primer) symbol		Primer sequence	T annealing (°C)
Elongation Factor 1 α (EF1α)	Fw	CGTCATAGTTTTCTGCCTTCTTCC	55
	Rev	TGCCACCGCCTATCAAGC	
Actin	Fw	GGTCAACCATGTTCCCTGGTATT	59
	Rev	GGAGCAAGAGCAGTGATTTCCTT	
HPL2	Fw	GAGAGGAAGCTTGCCACAAC	58
	Rev	AGACTTCATCAGCGGCATCT	
HPL5	Fw	CTTCTTCCTCTCTTCCCCTCA	58
	Rev	AGAACTGGTCACGGCCTTC	
HPL6	Fw	CGAGGCAGACTTCAATGACA	58
	Rev	TTGACGGTAAGGGAAAGGTG	
ADH1	Fw	GGTCAAGTCATCTGCTGCAA	58
	Rev	CGAAAATTCGAGGGAACAAA	
ADH2	Fw	GCGTTGAGTGTACCGGAAAT	58
	Rev	TTTCCACCACTGAAGGAAGG	
ADH3	Fw	AGAGGACTCTCAAGGGCACA	58
	Rev	TCCCCCTTCAGCATGTAGTC	
LOXA	Fw	GCAAATCAAAGGGACAACGCTGTATGG	58
	Rev	TGCTTCCACTGCGGCTTCC	
LOXC	Fw	TGGTGGAAGGAAGTCAGGGAAGAG	58
	Rev	TGGGCGGTTTGGGAGGTAGC	
LOXD	Fw	ACCCACCAAATCGTCCCACACTATG	58
	Rev	ACCTCTTCGTTGTCTGTCCACTCTG	
GS6	Fw	ACCCTTCAGCAACTCCACAG	58
	Rev	TCCAGGTTCACACAAGGAGA	
GS9	Fw	GGAGTGGGGAACCTATTCCT	58
	Rev	CATGTCCTCAATTCGTGCAT	
GS16	Fw	CAAGGGCAAACTCTTTTTGG	58
	Rev	ATTGACCATGCGAAGTACCC	
GS21	Fw	TCTTCAATGGAAGCTGCTGA	58
	Rev	CTATTCCCATCCGGAACACA	
GS25	Fw	AATATTGCCTGGGTGTTGGA	58
	Rev	TTGGAACCCCATCTCTTTCTC	
OMT1	Fw	CTCCGCATAGCCGATATCAT	58
	Rev	GAGAGTTTCTCCGCCATCTG	
OMT2	Fw	ACAAAGGAGTGTCCCACGTC	58
	Rev	GGCTCATAGCCATCTTCTCG	

2.5.5. Real-time quantitative PCR

The cDNA from the eight samples (C1, C2, C3, C4, I1/2, I3/4, I5/6 and I7) to be analyzed was diluted 40X with RNase free water. Before the final RT-qPCR essays, all primers (table 2.1.) were tested using two technical replicates of each sample. The first wells of the plates were used to the standard curve, the remaining wells were used for the samples and for the negative control. The standard curve was built using serial dilutions (1:1, 1:5, 1:25, 1:125 and 1:625) of mixtures of all cDNAs analyzed, in each one having three replicates. The samples used in RT-qPCR analysis were prepared with 1:10 dilution factor, with one technical replicate and one biological replicate to each sample. A master mix was prepared, so that each well would contain 10 µL of Syber Green, 0.7 µL of each primer (*forward* and *reverse*, 10 µM) and 4.6 µL of ultra-pure water and at last 4 µL is added in each well corresponding to the cDNA samples and standard curve dilution series. The final concentration was 20 µL. Real-time PCRs were performed using the StepOneTM Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 95 ºC for 10 min, followed by 42 cycles of 95 °C for 15 s and 56 ºC - 60 °C for 40 s. Primer efficiencies in between 85 and 105%, were calculated using 4-fold cDNA dilutions (1:1, 1:5, 1:25, 1:125, and 1:625) in triplicate as well as checking for amplification in a negative control without cDNA.

In order to obtain relative expression of the genes under study, data were normalized using the expression curves of the actin gene (VIT_04s0044g00580) and elongation factor 1α gene (VIT_06s0004g03220). These genes are the most stable according to NormFinder software (Agudelo-Romero *et al.* 2015).

2.5.6. Relative quantification methods

In order to assay the best candidate genes for further study, a previous RT-qPCR analysis was performed to all genes (table 2.1). Target genes were then selected based on a high or a low fold change between infected and control samples. The method $2^{-\Delta\Delta CT}$ was used to determine the relative expression of the target genes (Livak and Schmittgen 2001). This relative quantification describes the variation in the expression of the target gene (non-infected /control and infected samples) relative to reference genes.

The Ct values from target genes were obtained by LinRegPCR ((11.0) version 7.4). and were imported into Microsoft Excel a spreadsheet program and then calculated the ΔΔCt and $2^{-\Delta\Delta CT}$ values. Cts from target genes are subtracted to the reference genes Cts from each corresponding non-infected (control) and infected samples to proceed the ΔΔCt calculations. The ΔΔCt was calculated by the subtraction of infected samples to non-infected (control) and with this result the $2^{-\Delta\Delta CT}$ values were obtained. In Appendices, Table 13 and Appendices, Figure 9 presents the ΔΔCt and $2^{-\Delta\Delta CT}$ results values from each gene tested. The final fold change between non-infected (control) and infected samples represents the gene expression levels due to the infection.

2.5.7. Statistical analysis

All the results are expressed as mean ± standard deviation (SD). Data were examined by the non-parametric Mann– Whitney *U* test using IBM®SPSS® Statistics version 25.0 (SPSS, Inc.) software, with a statistically significant of $p \leq .05$.

2.6. Melatonin: Exploratory study

Grapevine berries Trincadeira and Touriga Nacional were treated with melatonin in August 2018 at the Instituto Superior de Agronomia, University of Lisbon, Portugal. Bunches of grapes were treated 9 days before harvest with 1 mM melatonin (with 3 treatments every 2-3 days) by spraying berry clusters with this hormone. Collection of Trincadeira (Appendices, Figure 2) and Touriga Nacional samples was performed at harvest stage of development, with 4 replicates from non-treated (controls) and treated bunches of grapes. They were briefly transported to the laboratory and frozen in liquid nitrogen and stored at -80 °C until further use.

2.6.2. Determination of total phenolic content

To study the total phenolic content from Trincadeira and Touriga Nacional berries treated with melatonin, have undergone the same process as described for *Botrytis* in section 2.3. To determinate the phenolic content in Trincadeira and Touriga Nacional with and without melatonin using water as extraction solvent, the normalization was made with the weight of approximately 25 mg of lyophilized grape berries (Appendices, Table 9) with the same standard curve of gallic acid as before.

2.6.3. Determination of total anthocyanin content

To study the anthocyanins from Trincadeira and Touriga Nacional berries treated with melatonin, they have undergone the same process as described for *Botrytis* in section 2.4. To determinate the anthocyanins content from Trincadeira and Touriga Nacional, the berries weight of approximately 25 mg of lyophilized grape berries was considered, with the weight tables in Appendices, Table 11.

3. Results

3.1. Identification and determination of free and glycosidic bound volatiles

Alcohol levels, such as ethanol, were relatively high in all Trincadeira grape chromatograms (Appendices, Figure 3), especially in infected grapes (Appendices, Figure 1). This behaviour can be explained by the fact that grapes had a higher maturation due to the fungus, originating more ethanol due to fermentative processes in grapes. Perestrelo *et al.* (2015) during his study discover that infected grapes have more quantity of ethanol than the non-infected (controls), indicating a possible spontaneous alcoholic fermentation. Since, ethanol is the main volatile compound found in chromatograms, it can mask the other compounds.

To better understand the difference between volatiles compounds with and without *B.cinerea*, the volatiles compounds percentage calculations were made with (Appendices, Table 3 and Figure 4) and without ethanol (Table 3.1 and Appendices, Figure 5). Concerning the fact that we want to study the volatile compounds (possible markers of the infection) that give us the information about the changes in aroma due to fungus and not due to fermentative processes. The free and glycosylated volatile composition is shown in Table 3.1 (without ethanol and its derivates), summarising the minimum and maximum percentages from each compound and grouped of compounds. The relative amount of each compound was calculated as the percent ratio of the respective peak area relative to the total peak area (RPA) and expressed as a percentage (%).

3.1.1. Free volatiles

In the experiments on free volatile fraction, 15 volatiles aroma compounds were identified (Table 3.1, Appendices, Figure 5). These compounds were classified based on their different biological synthesis: C_6 alcohols and aldehydes, amino acids derived esters and alcohols and alkanes. The most abundant grouped of compounds in infected grapes are C_6 alcohols and aldehydes with 77.9%. The non-infected (control) have a bigger percentage (79.6%) with 2-*trans*-hexenal as the main compound, followed by *n*-hexanol and hexanal. The infection increased the *n*-hexanol as the main compound and *cis*-2-hexen-1-ol. The amino acids derived esters and alcohols start their formation upon grapes infection. According to the results, only infected grapes have these compounds, such as *n*-nonanol, phenyl ethyl alcohol, 2-phenyl ethyl acetate and hexyl acetate with 14.4%. The non-infected (controls) can have some phenyl ethyl alcohol with low concentration. The alkanes appeared only in free volatile compounds with a total of 20.4% for non-infected (controls) and 7.7% for infected. In controls *n*-nonadecane (C_{19}) is the most abundant compound followed by *n*-eicosane (C_{20}) and *n*-octadecane (C_{18}). On infected grapes, *n*-nonadecane (C_{19}) is the main compound but, in controls exist in smaller quantity.

3.1.2. Glycosidic bound volatiles

In the glycosidic bound volatile fraction of Trincadeira, a total of 22 compounds belonging to the C_6 alcohols and aldehydes, amino acids derived esters and alcohols and fatty acids were identified. The glycosylated volatile composition is shown in Table 3.1. These included two C_6 alcohols and aldehydes, five fatty acids and fifteen amino acid derived esters and alcohols compounds. Controls have 17 compounds and infected grapes have 21 compounds detected.

Table 3.1 – Minimum and maximum percentage range (% of volatiles components identified in the free and glycoconjugated volatile isolated by SPME GC and GC-MS from non-infected (control) and infected Trincadeira grapes juices without the fermentative compounds (ethanol and its derived compounds), collected in ISA, during harvest stage.

		Free Forms						**Glycosidic Forms**					
		Trincadeira Control			Trincadeira Infected			Trincadeira Control			Trincadeira Infected		
Components	RI[c]	Min	Max	SD	Min	Max	SD	Min	Max	SD	Min	Max	SD
Ethanol	493												
Isopropyl Alcohol	495												
Acetaldehyde	497												
Ethyl acetate	603												
Acetic acid	606												
Isoamyl alcohol	722												
Amyl alcohol	839												
Hexanal	840	t[b]	20.6	7.8	t	41.5	15.2				t	3.5	1.1
2-*trans*-Hexenal	866	44.9	47.5	6.8	t	40.3	14.2						
***cis*-2-Hexen-1-ol**	882	t	8.2	4.1	t	27.4	10.9						
***n*-Hexanol**	882	12.1	23.4	4.1	33.5	70.1	12.55	t	33.5	10.6	6.6	15.2	2.6
Isoamyl acetate	882										6.7	21.5	4.3
2-Methyl butyl acetate	882				t	t	t	t	t	t	2.9	13.7	3.1
Hexanoic acid	970										t	3.0	0.9
Ethyl hexanoate	965							t	5.1	2.0	0.5	2.2	0.4
Hexyl acetate	995				t	3.0	1.1	t	t	t	3.4	8.1	1.9
Phenyl ethyl alcohol	1064	t	t	t	t	14.3	5.3	t	3.9	1.7	1.2	9.5	2.4
***n*-Nonanol**	1148				t	15.5	5.7	t	5.5	2.2	2.7	7.3	1.4
Octanoic acid	1149										t	1.0	0.4
Ethyl octanoate	1177				t	t	t	3.0	9.4	2.2	1.6	3.5	0.7
2-Phenyl ethyl acetate	1222				t	13.0	6.1	t	t	t	1.3	11.4	2.8
Ethyl nonanoate	1273							t	5.5	2.0	t	1.5	0.5
Nonyl acetate	1300							t	t	t	1.4	7.2	1.9
Decanoic acid	1356							t	t	t	t	15.4	4.7
Hexyl hexanoate	1375							t	2.7	1.1			
Ethyl decanoate	1387							23.1	44.4	7.4	7.1	24.8	5.1
Dodecanoic Acid	1550										t	7.3	2.4
Ethyl dodecanoate	1580							15.6	43.9	9.7	6.1	12.8	2.0
Ethyl tetradecanoate	1774							t	3.6	1.3	t	2.4	0.7
***n*-Octadecane (C_{18})**	1800	t	6.6	2.9	t	3.1	1.2						
***n*-Nonadecane (C_{19})**	1900	5.3	40.6	14.4	t	10.5	3.8						
Ethyl hexadecanoate	1936							1.8	7.8	1.9	2.5	11.9	2.8
***n*-Eicosane (C_{20})**	2000	t	5.6	2.7	t	5.7	2.5						
***n*-Heneicosane C_{21})**	2100	t	t	t	t	7.3	2.7						
Linoleic acid	2137							t	4.2	1.5	2.1	10	3.0
***n*-Docosane (C_{22})**	2200	t	t	t	t	t	t						
% of identification - Grouped Components													
C_6 alcohols and aldehydes		59.4	89.1		62.0	100		t	33.5		6.6	15.2	
Fatty acids								t	4.2		3.3	26.8	
Amino acid derived esters and alcohols		t	t		t	29.9		62.8	100		61.2	87.7	
Alkanes		10.9	40.6		0.0	12.6							
Others													

RI – linear retention index (relative to C_9–C_{22} n-alkanes); Min - minimum percentage; Max - maximum percentage; SD - standard deviation values

[b] t: traces (<0.05%)

[c] Retention indices according to (Kováts 1958)

The C_6 alcohols and aldehydes have a total of 15.8% for non-infected (controls) with *n*-hexanol and 10.3% for infected for *n*-hexanol and hexanal (Appendices, Figure 5).

Amino acid derived esters and alcohols have 82.0% for non-infected (controls). Ethyl decanoate and ethyl dodecanoate are the main volatiles (Maoz *et al*. 2018). While the infected have 77.5% with ethyl decanoate, isoamyl acetate, ethyl dodecanoate, ethyl hexadecanoate, 2-methyl butyl acetate, 2-phenyl ethyl acetate and hexyl acetate as the main compounds. Maoz *et al*. (2018) reported that 2-phenyl ethyl acetate wasn´t found in fresh fruit, maybe, for this reason, it only appears as consequence of grapes infection.

Fatty acids have a small number (2.2%) of non-infected (controls) volatile compounds and, 12.2% to infected. Comparing to the controls, infected grapes have all the compounds detected, beginning with decanoic acid and linoleic acid as the main volatile compounds followed by dodecanoic acid, hexanoic acid and octanoic acid. For non-infected (controls) they have linoleic acid and decanoic acid.

3.2. Determination of total phenolic content with *B.cinerea*

The phenolic content of grapes is very dependent on several parameters such as grape variety, maturity, variations in water and nutrient availability, light, temperature environment, changes in predation and disease stresses (Rambla *et al*. 2016). The phenolics compounds reported in grapes are tannins, flavan-3-ols, anthocyanins, hydroxycinnamates and flavonols (Ali *et al.* 2011).

To determine the phenolic content in Trincadeira with and without *Botrytis* (Infected and Control) was used two different extraction solvents, water and methanol. Described in previous reports, the decay of phenolic content occurs during the growth of Trincadeira berries (Ali *et al.* 2011). This decline since *veraison* to harvest occur due to the catabolism of phenolic compounds and their use in the synthesis of other compounds such as anthocyanins, flavonoids and dilutions from grape berry expansion (Ali *et al*. 2011). However, we can´t see all the development stages, because we are studying only the EL38 stage. At this ripening stage, the phenolic compounds and anthocyanins are deteriorated by *B.cinerea* (González-Barreiro *et al*. 2015).

The results in figure 3.1. (Appendices, Table 5), shows a decay of phenolic content in infected Trincadeira grapes compared to controls. Thus, these results reflect what other studies concluded, that infected grapes suffer a rapid maturation giving a faster deterioration due to the fungus, decreasing the phenolic content at this stage. Each method with methanol and water shares the same results, in both infected grapes, phenolic compounds decrease compared to controls as showed in figure 3.1 (Appendices, Table 5).

The only difference between methods is that using methanol as an organic solvent we can extract a little more phenolic content than with water. Because, phenols are more soluble with polar solvents such as methanol extracting more compounds. The best extraction solvent is methanol, compared to water. However, in water we have more significant variations between samples than with methanol. To finish this analysis, we can conclude that reducing the phenolic content, *B.cinerea* infection really change the grapes profile and quality, due to the increase of grapes maturation and tissues destruction.

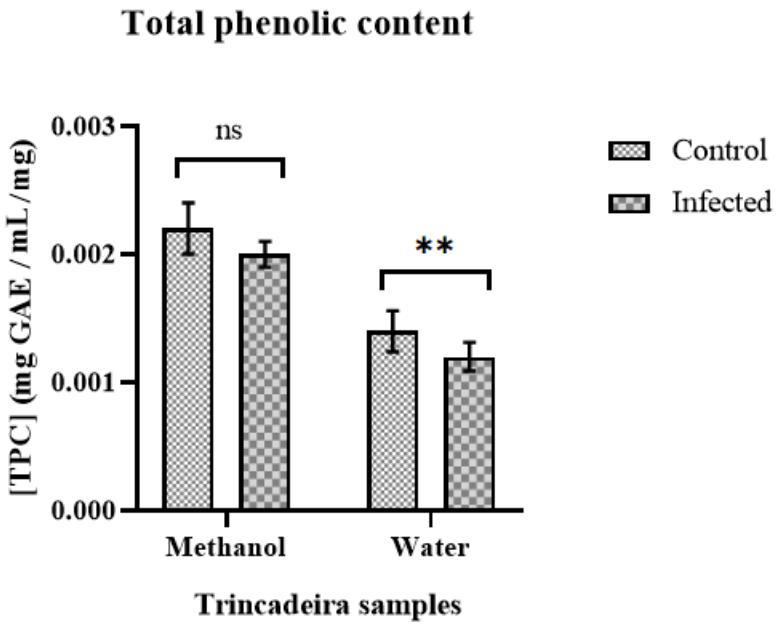

Figure 3.1. – Total phenolic content (TPC) expressed as mg GAE/mL gallic acid equivalent per mg of lyophilized grapes of non-infected (controls) and infected Trincadeira samples with methanol and water as extraction solvents. Results are expressed as mean ± standard variation (SD). Error bars represent SD. Data were analysed with Student´s t- test ($P < 0.05$): for each compound, 'ns' means none significant ($P > 0.05$) difference between the different samples, while '**' indicates more significant differences between samples ($P \geq 0.001$ to 0.01).

3.3. Determination of total anthocyanin content

Starting at *veraison* until harvest the accumulation of anthocyanins restricted to skin tissue and sugar levels increase (Ali *et al.* 2011). As expected, anthocyanins were more detected in control than in infected grapes, as shown in Figure 3.2. and Appendices, Table 7. This result is comparable to the ones found in other studies, like the one found in González-Barreiro *et al.* (2015) or Coelho *et al.* (2019), where the anthocyanin content is less in the infected grapes, due to the deterioration of the fungus as said before in the phenolic content. In this study, there were some significant variations between controls and infected grapes.

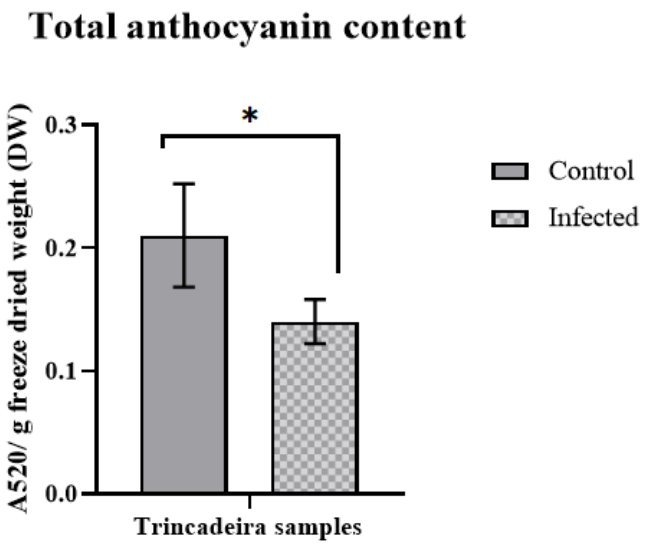

Figure 3.2.– Total anthocyanin content expressed as absorbance at 520 nm per g of freeze-dried (DW) material of non-infected (controls) and infected Trincadeira with *Botrytis cinerea*. Results are expressed as mean ± standard variation (SD). Error bars represent SD. Data were analysed with Student´s t- test ($P < 0.05$): for each compound, '*' indicates significant differences between samples ($P \geq 0.01$ to 0.05).

3.4. Expression levels of aroma genes from Trincadeira

To secure the integrity and quality of RNA samples extracted and purified, several electrophoreses in agarose gels were made. The finals gels of eight RNA samples from Trincadeira non-infected (control) samples (1, 2, 3, 4) and infected samples (1+2, 3+4, 5+6, 7) with *Botrytis cinerea* are shown in figure 3.3., and the rest in the Appendices, Figure 6. The respective RNA quantification after extraction and purification from 1 µL of eight samples analysed in NanoDrop® Spectrophotometer ND-1000 are in Appendices, Table 8. Furthermore, the RNA purified, and the respective quantification and quality allow us to proceed to reverse transcription.

The expression levels of genes related to aroma of Trincadeira were analysed by RT-qPCR and the results are in figure 3.4. The free volatile related genes *hydroperoxide lyase 2 (HPL2)* and *lipoxygenase C (LOXC)* are the ones with expression levels different between non-infected (control) and infected. Both genes had higher expression levels in response to the infection. The expression levels from *glutathione synthetase 21 (GS21)* and *O-methyltransferases 1 (OMT1)* genes were also considerable, but no significant fold changes were detected between non-infected (control) and infected in comparison to the *HPL2* and *LOXC* genes. Significant differences between non-infected (control) and infected samples were found only in HPL2 gene, with more expression in infected grapes. These results confirm the change in some grape aroma genes when infected with *B. cinerea*.

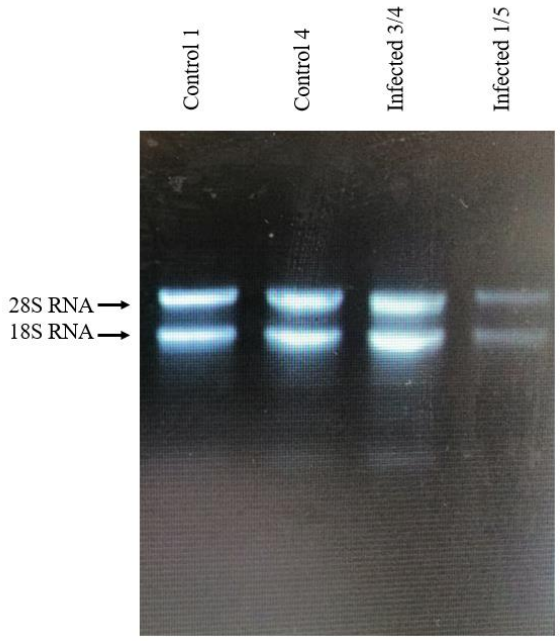

Figure 3.3.– Agarose gel electrophoreses showing the assessment of four RNA samples integrity from Trincadeira non-infected (control) and infected with *Botrytis cinerea*. Purified RNA was run through 1.1% agarose gel in 1 x TAE buffer. The agarose gel contained 3 µL of GelRed® nucleic acid gel stain. Samples on left are controls and on right are infected.

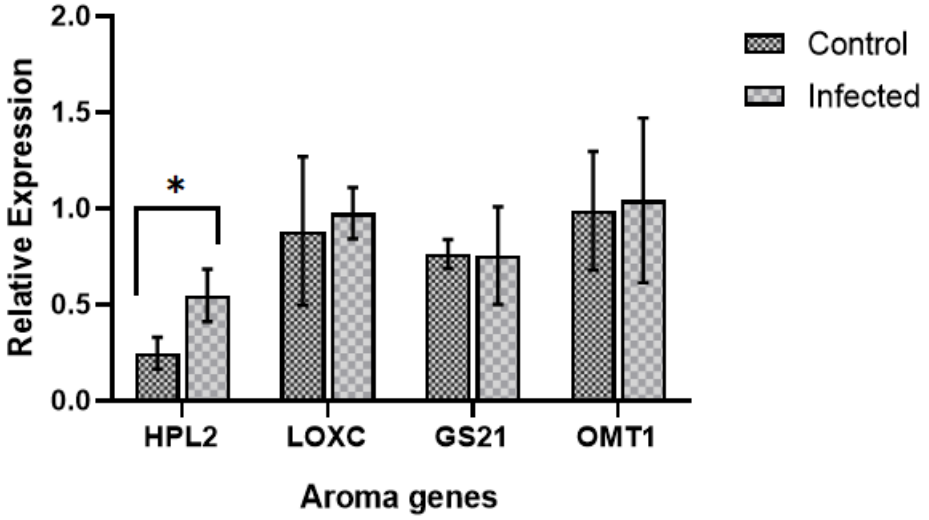

Figure 3.4. - Real time qPCR validation of the expression profiles of four genes (*HPL2, LOXC, GS21, OMT1*) before (control) and upon *Botrytis cinerea* infection of Trincadeira cultivar berries at harvest stage of fruit development (EL38). Data are reported as means ± standard deviation (SD) of one technical and biological replicate of each sample. Expression levels were calculated using the standard curve method and normalized against grapevine actin gene (VIT_04s0044g00580) and elongation factor 1α gene (VIT_06s0004g03220) used as reference controls. Asterisks (*) represent statistical significance ($p \leq .05$) of gene expression between the control (non-infected) and infected grapes, determined by the non-parametric Mann– Whitney *U* test using IBM®SPSS® Statistics version 25.0 (SPSS, Inc.) software.

3.5. Melatonin: Exploratory study

3.5.1 Determination of total phenolic and anthocyanins content with melatonin

Melatonin is a hormone found in grapes (Meng *et al.* 2018). This hormone helps plants to grow (Lili *et al.* 2018) and have a protecting role during the damage caused by various biotic and abiotic stresses like the presence of fungus *B.cinerea* (Meng *et al.* 2015). Many studies have shown that melatonin exists in the skin during *preveraison* decreasing during *veraison*, and in seeds and flesh increase since *preveraison* to *veraison* of grape berries (Meng *et al*. 2017). Melatonin has been assumed that can maximize the properties attributed to phenolic and others compounds (Meng *et al*. 2017).

This exploratory study, where exogenous melatonin was added to grapes to characterise the phenolic and anthocyanin content will give some evidences to understand if melatonin can increase or maintain these compounds. Touriga Nacional is known to have more phenolic compounds and anthocyanins than Trincadeira (Agudelo-Romero *et al*. 2013). The phenolic content of Touriga Nacional was higher in controls. During the treatment with melatonin somehow the phenols decreased. Trincadeira compared to Touriga have opposite results, grapes treated with melatonin had an increase of phenolic content and the controls had less, showing significant differences between them. The phenolic content upon melatonin treatment is shown in the Appendices, Figure 7 and Table 10. The anthocyanins in Touriga Nacional had the same results as the phenols in the treated grapes decreased. While Trincadeira maintain the same anthocyanins result in both controls and treated grapes. Anthocyanins results are shown in Appendices, Figure 8 and Table 12. Both varieties are different in some characteristics, and for this reason the results from this study can be distinct. Besides, without more research is still not clear (Lili *et al.* 2018) if melatonin has the effect of helping grapes during their growth, quality (C. Liu *et al*. 2018) and against possible infections with pathogens. Another study should be conducted to verify if melatonin can help grapes when they are damage by pathogens like *Botrytis*.

4. Discussion

4.1. Identification and determination of free and glycosidic bound volatiles

The literature described that C_6 fatty acids compounds have antifungal activity, defending the grapes against *B. cinerea* (Furdíková *et al.* 2019). Six carbon compounds are abundant volatiles in grape berries and usually named Green leaf volatiles (GLVs) and they give the green and herbaceous aroma in grapes (Cabaroglu *et al*. 2002). Previous results and studies from OuYang *et al.* (2015) shown that free forms volatiles have a high concentration of these compounds, specially, *n*-hexanol, *cis*-2-hexen-1-ol, 2-*trans*-hexanal and hexanal. The C_6 alcohols and aldehydes, hexanal, 2-*trans*-hexenal, *cis*-2-hexen-1-ol and *n*-hexanol are produced by enzymatic oxidation of linoleic and linolenic acids through grape crushing before fermentation (Panighel and Flamini 2014). They increase during the harvest stage, providing fruity, green and leafy notes, detected during the free form and reduced at the final maturation point or during the glycosylation process (Agudelo-Romero *et al.* 2013). In this study C_6 alcohols and aldehydes were found in the form of free volatiles and the percentage didn´t change much between non-infected (controls) and infected. However, some compounds increased during the infection (*n*-hexanol and *cis*-2-hexen-1-ol), while the non-infected (controls) have 2-*trans*-hexenal as the main compound, followed by *n*-hexanol and hexanal.

Alkanes (*n*-nonadecane (C_{19}), *n*-eicosane (C_{20}) and *n*-octadecane (C_{18})) are mainly free volatile compounds, and during the infection they decreased. This reduction maybe occurs due to an alteration of biosynthesis of these compounds with the infection.

Fatty acids volatiles (decanoic acid, linoleic acid, dodecanoic acid, hexanoic acid, octanoic acid and others) from Trincadeira appeared in the glycosidic form. Previous observations made by Agudelo-Romero *et al.* (2013) suggests the improvement of fatty acids biosynthesis. In line with this, fatty acids volatiles only arise during the glycosylation, increasing more when grapes are infected due to probably of a faster maturation, very similar to fermentations conditions (Panighel and Flamini 2014) and degradation of tissues by *B. cinerea*. The non-infected (controls) had linoleic acid and decanoic acid as the main volatiles detected.

Concerning the analysis of amino acids derived esters and alcohols during free form volatiles, only the infected grapes had some amino acids derived esters and alcohols volatiles such as *n*-nonanol, phenyl ethyl alcohol, 2-phenyl ethyl acetate and hexyl acetate. Once they are only produced during the infection, these compounds can be assumed markers of the infection. Through the glycosidic form, both controls and infected had high percentage of amino acid derived esters and alcohols volatiles. The non-infected grapes have ethyl decanoate and ethyl dodecanoate as the main volatiles. The infected grapes have ethyl decanoate, isoamyl acetate, ethyl dodecanoate, ethyl hexadecanoate, 2-methyl butyl acetate, 2-phenyl ethyl acetate and hexyl acetate as the main compounds. Some studies from Ali *et al.* (2011) show that usually Trincadeira have a smaller amount of amino acids derived esters and alcohols volatiles. In this present study, Trincadeira have only a small amount of amino acids derived esters and alcohols volatiles during their free form when infected. However, these compounds increase in their glycosidic form, in both control and infected grapes.

Normally with the infection of *B. cinerea* esters such as 2-phenyl ethyl acetate (with a sweet, honey, rosy aroma, located in berry skins) and fatty acids like decanoic and dodecanoic acid increase, or new ones are formed as consequence of grapes infection (Maoz *et al.* 2018). Some compounds such as, 2-phenyl ethyl acetate, ethyl octanoate and hexyl acetate in literature are the compounds reported with the highest

concentration in infected grapes (Perestrelo *et al.* 2015). The compounds from infected grapes found in the literature are the same found in this study. Ethyl hexanoate, ethyl octanoate, ethyl decanoate, hexanoic acid and octanoic acid in Slegers *et al.* (2015) are reported to be dependent of fermentation conditions such as yeast strain and temperature. Grapes have a process close to fermentation when *β*-glycosidase enzyme and *Botrytis* are added exogenously, accelerating the grape maturation more than usual, and therefore increasing these compounds.

According to previous studies from Cabrita *et al.* (2007a) and Freitas *et al.* (2006), Trincadeira is a neutral variety or not aromatic. Furthermore, this assumption is true, because wasn´t found any terpenic or benzenoic compounds in this present study. Trincadeira grapes are not aromatic (Freitas *et al*. 2006), but after the fermentative process the wine produce aromatic volatile compounds. For this reason, it was possible to see more volatiles adding *β*-glycosidase enzyme to the grape juices, inducing the glycosidic volatiles form. Adding the exogenous *β*-glycosidase to grape juice, occur a rapid acceleration and release of free aglycones, producing odor-active volatiles (Zhu, Du, and Li 2014) as the potential aromatic compounds in grapes induced by the hydrolysis of this enzyme. Producing an enzymatic hydrolysis, we have some glycosidic precursors from grape pulp and skin comparable to others aromatic grapes varieties or wine (Cabrita *et al.* 2007). Overall, glycosidic volatiles form comparing to free volatiles form, have more volatiles compounds and some of them don´t exist or are present in minimal quantities (t = trace, Table 3.1.) in the free volatile form. The volatiles compounds found only in the glycosidic bound form are the isoamyl acetate, 2-methyl butyl acetate, hexanoic acid, ethyl hexanoate, octanoic acid, ethyl octanoate, ethyl nonanoate, nonyl acetate, decanoic acid, hexyl hexanoate, ethyl decanoate, dodecanoic acid, ethyl dodecanoate, ethyl tetradecanoate, ethyl hexadecanoate and linoleic acid. Hexyl hexanoate in the glycosidic bound volatiles just appeared in controls. Most of the volatile compounds in the glycosidic bound form came from the grape itself. The volatile compounds formed due to infection are hexanal, isoamyl acetate, hexanoic acid, octanoic acid and dodecanoic acid. These five volatile compounds can suggest some information about the infection: *Botrytis* increased even more the production of these volatiles or this increase might occur to own protection of grapes against this pathogen.

Each grapevine aroma is different due to their profile of volatiles compounds. This difference is mostly due to the level variation of the volatile compounds that constitute the aroma of grape and not to the qualitative differences between volatile compounds. Generally free volatiles forms are more abundant in skins than in pulp or juice (Gunata *et al.* 1985), giving the green aroma. The glycosidic volatiles are more abundant than free volatiles and are highly associated to grape maturity, one of the reasons why the concentration of aromatic alcohols increase. Besides, this is also important since each volatile compound produced in grapes is derived from different berries tissues (Wu *et al*. 2016). The knowledge of grape ripening process and the volatile compounds produced specially with the infection will provide an advantage to a sustainable production of high-quality grapes and wine production.

4.2. Expression levels of aroma related genes from Trincadeira with and without *Botrytis cinerea*

Trincadeira variety presents a certain type of volatile compounds giving to grapes and wine a characteristic aroma profile. A total of 29 volatiles compounds were identified in previous SPME GC-MS analysis, belonging to different metabolic pathways in order to produce the final aroma of Trincadeira grapes.
A set of primers, for studying gene expression of LOX, HPL, ADH, GS and OMT enzymes were selected

based on the volatile compounds detected in previous SPME GC-MS analysis and from Rambla *et al.* (2016) studies to determinate their expression levels in Trincadeira samples through RT-qPCR. Each gene was study to determine specific volatile fractions: *LOX, HPL* and *ADH* belongs to the free volatile fraction; *GS* and *OMT* belongs to glycosidic fraction.

Cellular tissue breakdowns in plants starts the enzymatic production of fatty acids derivatives (Rambla *et al*. 2016) such as C_6 aldehydes and alcohols (hexanal, 2-*trans*-hexenal, *cis*-2-hexen-1-ol and *n*-hexanol), responsible for the "green" aroma in grapes (Vilanova *et al*. 2013). The infection with *B. cinerea* will contribute to a higher increase of tissues disruption releasing more C_6 aldehydes and alcohols volatile compounds.

From linoleic acid (detected in the glycosidic fraction samples), hexanal and *cis*-2-hexen-1-ol are formed due to the action of a lipoxygenase (LOX) followed by a hydroperoxide lyase (HPL) and reduced to their corresponding alcohols (1-hexanol and *cis*-2-hexen-1-ol) by the action of an alcohol dehydrogenase (ADH) when grape is crushed (Rambla *et al.* 2016).

Data from Rambla *et al*. (2016) suggests that *LOXA, HPL2* and *HPL6* from linoleic acid produce 2-*trans*-hexenal. *ADHs* have a high correlation to lipid derivates and can be related to the ethylene signalling pathway (Rambla *et al*. 2016).

The results in figure 3.4. show that *HPL2* from all the HPLs genes analysed is the gene with higher expression levels and better statistical significance between non-infected (control) and infected samples. *LOXC* proves to be the one from *lipoxygenases (LOXs)* with good expression levels in Trincadeira. During the SPME GC-MS analysis was detected some C_6 aldehydes and alcohols (hexanal, 2-*trans*-hexenal, *cis*-2-hexen-1-ol and *n*-hexanol), increasing more their number when grapes were infected. The results from RT- qPCR analysis support the volatiles compounds increase due to the infection, since the expression levels from *HPL2* and *LOXC* also increased.

The conclusion is that occurred a huge acceleration of C_6 aldehydes and alcohols volatiles production and release in Trincadeira due to *B. cinerea* attack, destroying more grape tissue leading to complete tissue death and changing the aroma profile over time.

A significant number of volatile compounds considered relevant for Trincadeira aroma are derived from amino acids. Most of them appears during the hydrolysis of grapes or due to the infection.

Amino acids compounds due to deamination, decarboxylation, reduction and esterification are converted into aldehydes, alcohols and esters (Rambla *et al.* 2016). The genes involved in the amino acid metabolism are the *glutathione synthetases (GSs).* The biosynthesis of these compounds starts with phenylalanine ammonia-lyase (PAL) producing (E)-cinnamic acid and ending with the canalization of these compounds by an *O*-methyltransferase (OMT) (Rambla *et al.* 2016).

The SPME GC-MS analysis revealed that amino acids derived esters and alcohols volatiles appeared during the infection and are glycosylated. Most of these volatiles are dependent on fermentation conditions as said before (Slegers *et al*. 2015), and with *β*-glycosidase and *Botrytis cinerea* the maturation of grapes accelerate more than normal. It is assumed that some of these volatiles can be markers of the infection. Furthermore, glutathione and *trans*-2-hexenal were identified as intermediates of *S*-(3-hexan-1-ol)-glutathione biosynthesis, also known for being a flavour precursor in wines (Agudelo-Romero *et al.* 2013).

The genes from Trincadeira aroma derived from amino acids for RT-qPCR were *GS21* and *OMT1*. Agudelo-Romero *et al*. (2013) described that *O-methyltransferase (OMT)* was only identified in grapes at EL36 and

EL38 stages of ripening. This present study confirms that *OMTs* are present during the EL38 stage with the *OMT1* results found in Trincadeira. The expression level of *OMT1* was higher during the infection comparing to controls, but not significant.

The expression of *glutathione synthetases (GSs)* increases during grape ripening when related to the higher oxidative stress, but are reduced by 90% in the presence of ascorbic acid due to a rapid oxidation to dehydroascorbic acid (Agudelo-Romero *et al.* 2013).

GS21 expression in infected grape presented considerable differences among biological replicates as indicated by the standard deviation. The result from RT-qPCR give us almost the same result to non-infected (control) and infected grapes. If we had more infected grapes (biological sample variety) to do this analysis probably this gene would demonstrate the reduction of *GS21* expression as described above comparing to non-infected (control).

Good correlations were found for the four genes associated to some volatile compounds involved in aroma profile of Trincadeira. The results from RT-qPCR in this experiment revealed what was found before in other studies and proving the previous data from SPME GC-MS volatiles found in Trincadeira grapes. The infection gives to grape difference in aroma.

5. Conclusion

The present work was carried out to characterize the changes in Trincadeira grape aroma when infected with *Botrytis cinerea.* Trincadeira is a common variety used to produce wine in Portugal. However, it is susceptible to *Botrytis cinerea* destroying the berries and changing their aroma and quality. Previous studies presented the aroma of tomato (Rambla *et al*. 2014), grapes Tempranillo, Airén (Rambla *et al*. 2016) and Trincadeira (Agudelo-Romero *et al.* 2013), but never with the infection of *Botrytis*. For this purpose, the Trincadeira grape aroma was studied through the volatiles compounds analysis by SPME GC-MS and the responsible genes of aroma by RT-PCR analysis.

The number of volatile compounds identified during the SPME GC-MS analysis increased in the glycosidic form as shown by the addition of *β*-glycosidase enzyme to the grape juice. It was found four groups of volatiles in grapes, the C_6 alcohols and aldehydes, alkanes, fatty acids and the amino acids derived esters and alcohols. The C_6 alcohols and aldehydes volatiles protect grapes when they are infected with *Botrytis*. In this study, was found that *n*-hexanol and *cis*-2-hexen-1-ol increased during the infection while 2-*trans*-hexenal appear in more quantity without the infection. Perhaps, this increase was a way to protect the grape against this pathogen. In general, the quantity of C_6 volatiles found in grapes infected and non-infected are very equivalent. The alkanes were found during the analysis of free volatiles form and in this study *n*-nonadecane (C_{19}), *n*- eicosane (C_{20}) and *n*-octadecane (C_{18}) decreased during the infection. *Botrytis* reduce the alkanes by changing the biosynthesis of volatiles compounds. The fatty acids volatiles appeared in the glycosidic form and increased even more during the infection. The compounds found during the analysis were mostly decanoic acid, linoleic acid, dodecanoic acid, hexanoic acid, octanoic acid and other volatiles. *Botrytis* increased these compounds due to a faster maturation and degradation of grapes. The amino acids derived esters and alcohols were found in the free and glycosidic volatile forms. In the free form analysis, the infected grapes had more *n*-nonanol, phenyl ethyl alcohol, 2-phenyl ethyl acetate and hexyl acetate volatile compounds. Since they only appeared during the infection, they can be considered markers of the infection. During the analysis of glycosidic form, the infected grapes had lesser quantity of volatile compounds compared with non-infected. Both had in high amount ethyl decanoate, ethyl dodecanoate in infected grapes and ethyl decanoate, isoamyl acetate, ethyl dodecanoate, ethyl hexadecanoate, 2-methyl butyl acetate, 2-phenyl ethyl acetate in non-infected. The volatile analysis confirms that Trincadeira variety is neutral or not aromatic.

The gene expression was studied through the analysis of LOX, HPL, *ADH,* GS and OMT enzymes. Only, the *LOXC, HPL2, GS21* and *OMT1* had interesting results. The *HPL2* had the highest expression levels and better statistical significance between non-infected and infected samples. *LOXC* was the other with differences in expression levels between the samples through not significant. Both genes had higher expression during the infection and belong to the biosynthesis of C_6 aldehydes and alcohols volatiles that increased also during the infection. *GS21* and *OMT1* had lesser expression levels in between samples compared to *LOXC* and *HPL2. OMT1* had more expression during the infection. However, *GS21* had the equivalent expression level between infected and non-infected samples. These genes belong to the biosynthesis of amino acids derived esters and alcohols volatiles, appearing during the highest stages of maturation of grapes and due to *β*-glycosidase enzyme or *Botrytis cinerea*. The results also suggest that changes in gene expression of aroma related genes probably occurred at an earlier stage of ripening and are not so evident at EL38 stage.

The phenolic content and anthocyanins analysis showed a decreased during the infection due to the deterioration of these compounds by *Botrytis cinerea*.

In conclusion, the infection with *Botrytis cinerea* presents to Trincadeira grapes many modifications in the biosynthesis of several enzymes that produce the volatile compounds of grape aroma, changing the aroma profile and the quality of grapes. This knowledge provides the ability to start to understand which volatile compounds are modified by the infection and their corresponding biosynthetic pathways. These are still preliminary findings which can provide a base for a future research related to changes in grape aroma when infected with *Botrytis cinerea*. In the future this information will provide an advantage to a sustainable production of high-quality grapes and possible ways to reduce the infection without applying harmful products to the environment.

6. Future prospects

The present study provides novel insights about the characterization of grape aroma when infected with *Botrytis cinerea*. However, this work leaves many questions unanswered which need to be investigated, to have a better understanding of grape aroma and how it responds to the infection:

- Repeating the volatile analysis of this present study during the next year with more biological replicates of grapes bunches infected will settle a better outcome to this preliminary study. It will be interesting to relate the level of infection with the corresponding amount of volatiles, but for this at least 10 infected clusters will be necessary. The level of infection will be assessed by visual inspection and molecular methods;

- Although gene expression levels revealed which enzymes are likely to change due to infection, it would be desirable to explore more genes and enzymes related to grape aroma and repeat again the same genes/enzymes in grapes during the next year to confirm the results found in this study;

- Since all grape varieties are different, for instance Syrah is resistant to *Botrytis cinerea* while Trincadeira is very susceptible. To further understand how Syrah is resistant it would be interesting to analyse the volatiles produced in each one of them and the expression levels of genes involved in the aroma. Eventually, volatiles also play a role in tolerance of grapes against fungal diseases;

- Even through the present study deals with the aroma of grapes, it would be interesting to understand how the enzymatic activities, hormones and other metabolites changes during the infection.

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8. Appendices

Appendices, Figure 1– Harvest stage Trincadeira bunch infected with *Botrytis cinerea* at Instituto Superior de Agronomia (ISA) in Lisbon and collected during 2018 ripening season. The infections were made by spraying berry clusters with a conidial suspension at the developmental stage of peppercorn size (stage EL29).

Appendices, Figure 2– Harvest stage Trincadeira bunches with melatonin treatment on right and without melatonin on left at Instituto Superior de Agronomia (ISA) in Lisbon and collected during 2018 ripening stage. Grapes were treated 8 days before harvest with 1 mM melatonin (with 3 treatments every 2-3 days) by spraying berry clusters with this hormone. No visible differences in phenotypes were observed.

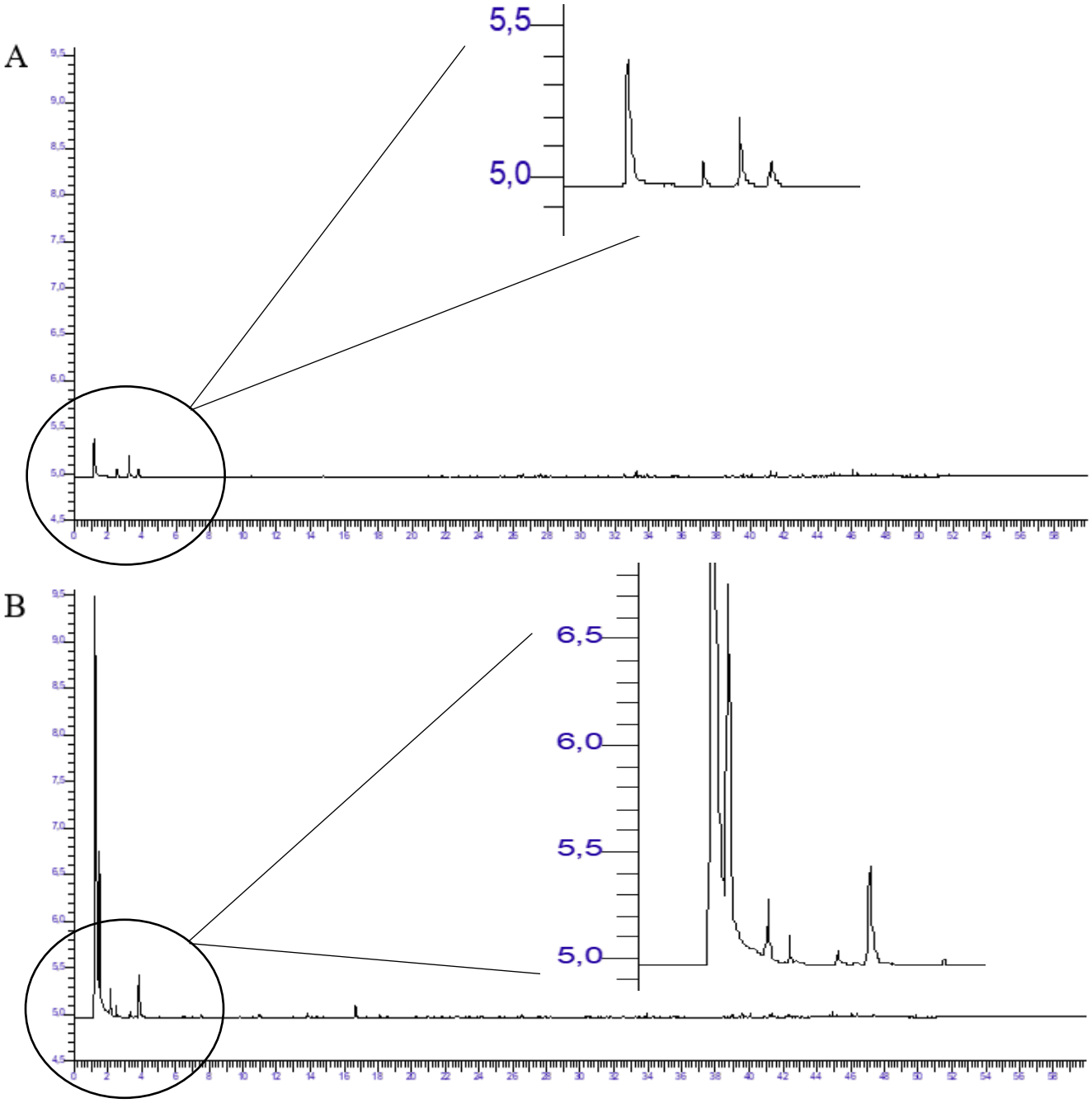
Appendices, Table 1. – Weight values (g) of Trincadeira grape bunches non-infected and infected with *B.cinerea*. Some heavy grape bunches were divided and weighted twice.

		Weight (g)	Second weight (g)
Non-infected	Control 1 (C1)	182.30	
	Control 2 (C2)	131.96	
	Control 3 (C3)	190.48	
	Control 4 (C4)	103.90	
Infected	Infected 1 (I1)	181.04	
	Infected 2 (I2)	114.09	107.10
	Infected 3 (I3)	175.13	182.34
	Infected 4 (I4)	161.88	
	Infected 5 (I5)	158.34	143.27
	Infected 6 (I6)	166.59	
	Infected 7 (I7)	176.71	165.12

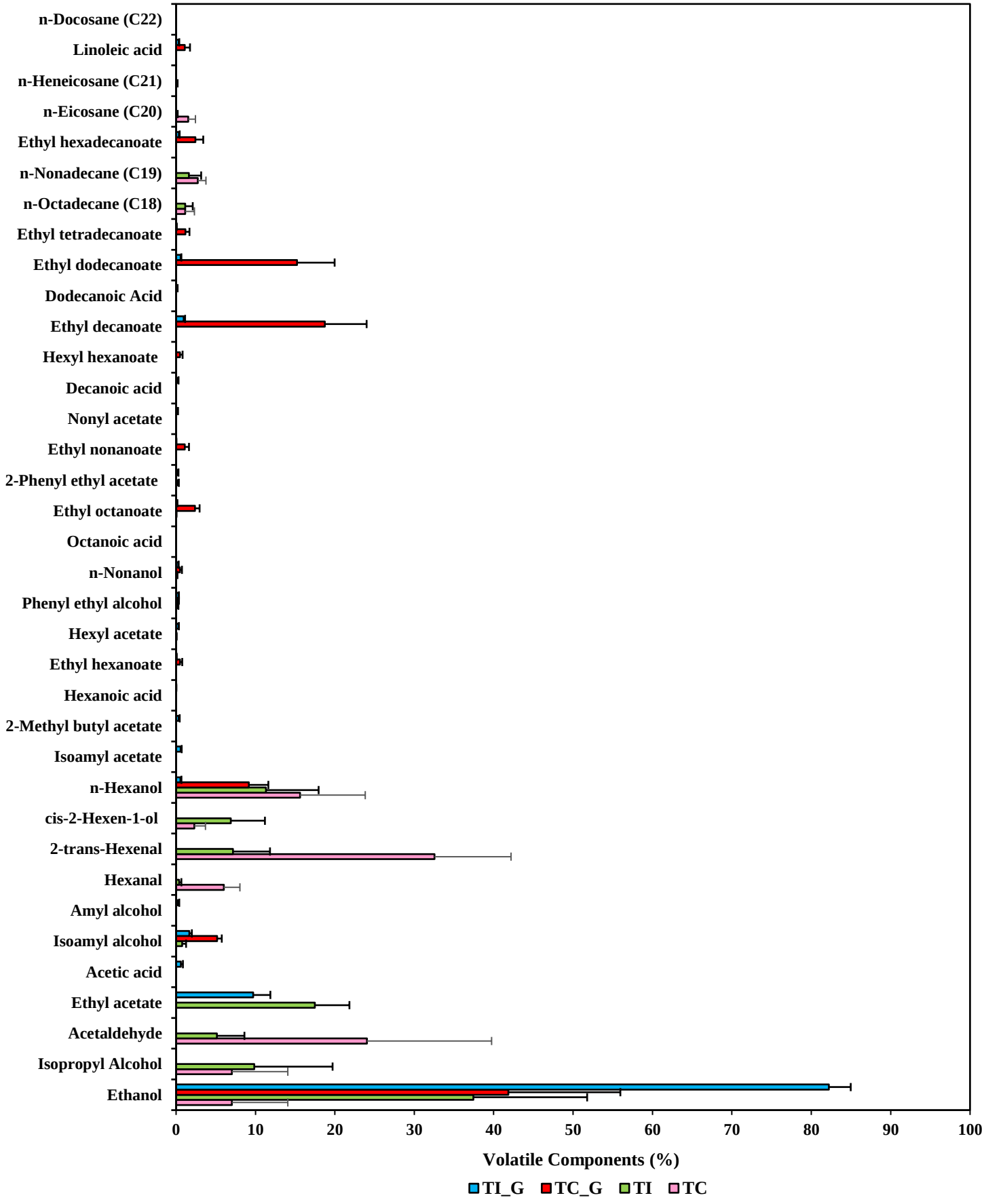
Appendices, Table 2 – Code names of SPME fibers with each date and volume from vials used during the analysis of free and glycosidic volatile (all vials with 15 mL).

		Free Volatiles				**Glycosidic Volatiles**		
		Date	GC	GC-MS	Volume of each vial (mL)	Data	GC	GC-MS
Non-infected	C 1	05/09/2018	TCTa_F10	TCTb_F12	15			
		05/09/2018	TCEa_F3	TCEb_F9	15			
	C 2					14/09/2018	TC2a_G_F7	TC2B_G_F3
						20/09/2018	TC2c_G_F4	TC2d_G_F9
	C 3	04/09/2018	TC3a_F9	TC3b_F4	3	07/09/2018	TC3a_G_F4	TC3b_G_F7
						24/09/2018	TC3c_G_F8	TC3d_G_F7
	C 4	04/09/2018	TC4a_F12	TC4b_F7	3	12/09/2018	TC4a_G_F7	TC4b_G_F9
						28/09/2018	TC4c_G_F7	TC4d_G_F14
Infected	Inf. 1	04/09/2018	TI1a_F3	TI1b_F10	3			
		05/09/2018	TI1Ta_F7	TI1Tb_F4	15			
		05/09/2018	TI1Ea_F3	TI1Eb_F8	15			
	Inf. 2	05/09/2018	TI2Ea_F8	TI2Eb_F9	10			
		05/09/2018	TI2Ta_F10	TI2Tb_F3	10			
	Inf. 3					12/09/2018	TI3a_G_F3	TI3b_G_F10
						02/10/2018	TI3c_G_F10	TI3d_G_F8
	Inf. 4					12/09/2018	TI4a_G_F8	TI4b_G_F9
						24/09/2018	TI4c_G_F9	TI4d_G_F4
	Inf. 5					07/09/2018	TI5a_G_F22	TI5b_G_F12
						02/10/2018	TI5c_G_F3	TI5d_G_F4
	Inf. 6					11/09/2018	TI6c_G_F3	TI6b_G_F8
	Inf. 7	04/09/2018	TI7Aa_F8	TI7Ab_F22	3	12/09/2018	TI7a_G_F8	TI7b_G_F10
						28/09/2018	TI7c_G_F4	TI7d_G_F3

T – Trincadeira; C(x) – control (x = control number); I(x) – infected (x = infected number); T - technical replica; E - biologic replica; F(x) – SMPE fiber (x = fiber number used); a, Aa and c – used in GC; b, Ab and d – used in GC-MS; G – glyosidic;



Appendices, Figure 3 - Examples of SPME-GC–MS chromatograms obtained of grapes free volatile compounds identification: Trincadeira control (A), Trincadeira infected (B). Peaks were identified as follow in Appendices, Table 3, from ethanol to n-docosane (C22).


n-Docosane (C22)
Linoleic acid
n-Heneicosane (C21)
n-Eicosane (C20)
Ethyl hexadecanoate
n-Nonadecane (C19)
n-Octadecane (C18)
Ethyl tetradecanoate
Ethyl dodecanoate
Dodecanoic Acid
Ethyl decanoate
Hexyl hexanoate
Decanoic acid
Nonyl acetate
Ethyl nonanoate
2-Phenyl ethyl acetate
Ethyl octanoate
Octanoic acid
n-Nonanol
Phenyl ethyl alcohol
Hexyl acetate
Ethyl hexanoate
Hexanoic acid
2-Methyl butyl acetate
Isoamyl acetate
n-Hexanol
cis-2-Hexen-1-ol
2-trans-Hexenal
Hexanal
Amyl alcohol
Isoamyl alcohol
Acetic acid
Ethyl acetate
Acetaldehyde
Isopropyl Alcohol
Ethanol
0
10
20
30
40
50
60
70
80
90
100
Volatile Components (%)
TI_G
TC_G
TI
TC

Appendices, Figure 4– Percentage (%) of specific volatiles compounds detected by SPME-GC-MS. Bar plot with standard error between samples of free volatiles in controls (TC) in samples infected with *B.cinerea* (TI), glycosidic volatiles in controls (TC_G) and glycosidic volatiles in infected samples (TI_G) from Trincadeira juice. The results are shown as the mean values considering ethanol in the analysis.

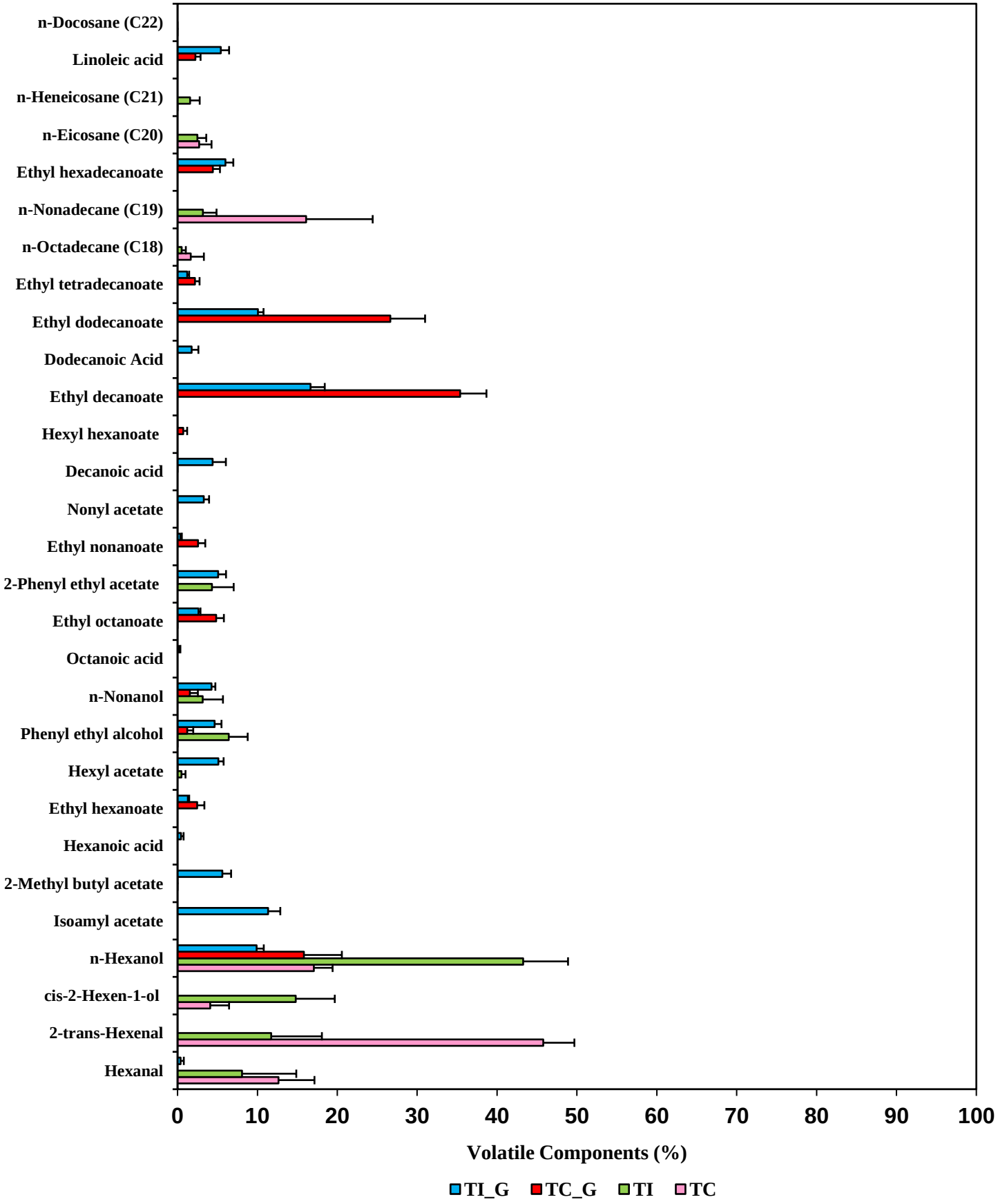
Appendices, Table 3.- Minimum and maximum percentage (%) of volatiles components identified in the free and glycoconjugated volatiles isolated by SPME GC and GC-MS from non-infected (control) and infected Trincadeira grapes juices with the fermentative compounds (ethanol and its derived compounds).

		Free Forms						**Glycosidic Forms**					
		Trincadeira Control			Trincadeira Infected			Trincadeira Control			Trincadeira Infected		
Components	RI[c]	Min	Max	SD	Min	Max	SD	Min	Max	SD	Min	Max	SD
Ethanol	493	t[b]	28.1	12.2	t	72.0	32.1	3.8	89.0	31.6	70.2	95.7	7.9
Isopropyl Alcohol	495	t	28.1	12.2	t	59.1	22.0				t	t	t
Acetaldehyde	497	t	66.0	27.2	t	20.2	7.8						
Ethyl acetate	603				t	27.1	9.8	t	t	t	1.9	18.4	5.8
Acetic acid	606	t	t	t	t	t	t	t	t	t	t	1.6	0.7
Isoamyl alcohol	722				t	2.8	1.1	3.2	7.5	1.3	0.2	3.0	0.9
Amyl alcohol	839										t	1.5	0.5
Hexanal	840	t	9.0	3.5	t	1.8	0.6				t	t	t
2-*trans*-Hexenal	866	17.8	60.2	16.7	t	26.3	10.4						
***cis*-2-Hexen-1-ol**	882	t	5.7	2.4	t	24.5	9.6						
***n*-Hexanol**	882	4.2	39.8	14.2	0.4	39.2	14.8	1.9	18.3	5.5	0.2	1.4	0.4
Isoamyl acetate	882										0.2	1.4	0.4
2-Methyl butyl acetate	882				t	t	t	t	t	t	0.1	1.3	0.4
Hexanoic acid	970										t	0.2	0.1
Ethyl hexanoate	965							t	1.6	0.6	t	0.1	0.1
Hexyl acetate	995				t	0.2	0.1	t	t	t	0.1	0.8	0.2
Phenyl ethyl alcohol	1064	t	t	t	t	0.5	0.2	t	1.0	0.4	t	0.7	0.2
***n*-Nonanol**	1148				t	0.4	0.2	t	1.5	0.6	0.1	0.7	0.2
Octanoic acid	1149										t	t	t
Ethyl octanoate	1177				t	0.2	0.1	0.3	4.5	1.3	0.1	0.3	0.1
2-Phenyl ethyl acetate	1222				t	1.0	0.4	t	t	t	0.1	0.4	0.1
Ethyl nonanoate	1273							t	3.2	1.2	t	0.1	0.1
Nonyl acetate	1300							t	t	t	t	0.5	0.2
Decanoic acid	1356							t	t	t	t	0.6	0.2
Hexyl hexanoate	1375							t	1.6	0.7			
Ethyl decanoate	1387							2.2	34.9	11.8	0.2	1.6	0.6
Dodecanoic Acid	1550										t	0.3	0.1
Ethyl dodecanoate	1580							1.4	29.5	10.6	0.2	1.0	0.3
Ethyl tetradecanoate	1774							t	3.2	1.1	t	0.2	0.1
***n*-Octadecane (C_{18})**	1800	t	4.6	2.0	t	5.9	2.1						
***n*-Nonadecane (C_{19})**	1900	t	4.4	1.8	t	9.49.4	3.5						
Ethyl hexadecanoate	1936							0.4	7.0	2.2	0.1	0.7	0.3
***n*-Eicosane (C_{20})**	2000	t	3.6	1.6	t	0.3	0.1						
***n*-Heneicosane (C_{21})**	2100	t	t	t	t	0.5	0.2						
Linoleic acid	2137							t	3.9	1.5	0.1	0.8	0.3
***n*-Docosane (C_{22})**	2200	t	t	t	t	t	t						
% of identification - Grouped Components													
C_6 alcohols and aldehydes		29.6	100		0.7	80.1		1.9	18.3		0.2	1.4	
Fatty acids								t	3.9		0.1	1.5	
Amino acidderived esters and alcohols		t	t		t	2.0		5.7	76.3		1.5	7.5	
Alkanes		t	12.4		0.1	9.4							
Others		t	66.0		10.6	99.1		9.2	92.3		90.7	98.0	

RI – linear retention index (relative to C_9–C_{22} n-alkanes); Min - minimum percentage; Max - maximum percentage; SD - standard deviation values

[b] t: traces (<0.05%)

[c] Retention indices according to (Kováts 1958)


n-Docosane (C22)
Linoleic acid
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Ethyl tetradecanoate
Ethyl dodecanoate
Dodecanoic Acid
Ethyl decanoate
Hexyl hexanoate
Decanoic acid
Nonyl acetate
Ethyl nonanoate
2-Phenyl ethyl acetate
Ethyl octanoate
Octanoic acid
n-Nonanol
Phenyl ethyl alcohol
Hexyl acetate
Ethyl hexanoate
Hexanoic acid
2-Methyl butyl acetate
Isoamyl acetate
n-Hexanol
cis-2-Hexen-1-ol
2-trans-Hexenal
Hexanal
0
10
20
30
40
50
60
70
80
90
100
Volatile Components (%)
TI_G
TC_G
TI
TC

Appendices, Figure 5 – Percentage (%) of specific volatiles compounds detected by SPME-GC-MS. Bar plot with standard error between samples of free volatiles in controls (TC) in samples infected with *B.cinerea* (TI) glycosidic volatiles in controls (TC_G) and glycosidic volatiles in infected samples (TI_G) from Trincadeira juice. The results are shown as the mean values without considering ethanol in the analysis.

Appendices, Table 4 – Weight values (g) of Trincadeira lyophilized grapes with and without *Botrytis cinerea* infection used in quantification of phenolic content by applying methanol and water as extraction solvent.

		Trincadeira Weight (g)	
	Samples	Methanol	Water
Controls	C1	0.0257	0.0260
	C1 r	0.0252	0.0249
	C2	0.0256	0.0257
	C2 r	0.0260	0.0256
	C3	0.0254	0.0252
	C3 r	0.0261	0.0258
	C4	0.0259	0.0262
	C4 r	0.0254	0.0257
Infected	I 1	0.0272	0.0259
	I 1 r	0.0267	0.0261
	I 2	0.0259	0.0255
	I 3	0.0262	0.0258
	I 4	0.0256	0.0260
	I 5	0.0254	0.025
	I 6	0.0259	0.0263
	I 7	0.0258	0.0257

Appendices, Table 5 - Total phenolic content (TPC) expressed as gallic acid equivalent (mg GAE / mL) per mg of lyophilized grape of Trincadeira samples non-infected (control) and infected[a].

Solvents used for extraction		Mean ± SD [Trincadeira] mg/mL / mg	Minimum (mg/mL / mg)	Maximum (mg/mL / mg)
Methanol	Controls	0.0022 ± 0.0002	0.002	0.002
	Infected	0.002 ± 0.0003	0.001	0.002
Water	Controls	0.0014 ± 0.00016	0.0012	0.0017
	Infected	0.0012 ± 0.00011	0.0010	0.0013

[a]Values expressed are mean ± SD (standard deviation) of two experiments

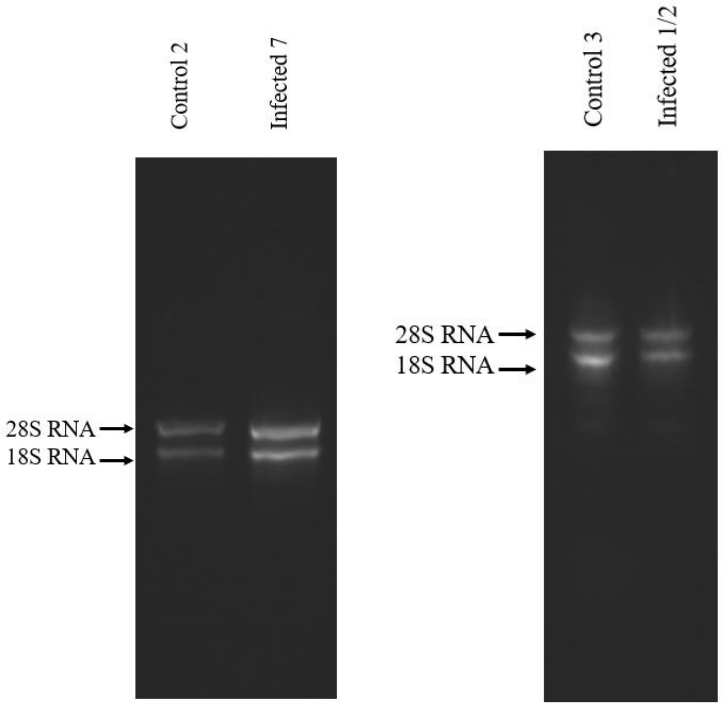
Appendices, Table 6 – Weight values (g) of Trincadeira lyophilized grapes with and without *Botrytis cinerea* infection used for quantification of anthocyanins.

		Weight (g)
	Samples	Trincadeira
Controls	C1	0.0504
	C2	0.0499
	C3	0.0502
	C4	0.0505
Infected	I 1	0.0509
	I 2	0.0496
	I 3	0.0504
	I 4	0.0498
	I 5	0.0502
	I 6	0.05
	I 7	0.0493

Appendices, Table 7 – Total anthocyanin content of controls and infected[a] Trincadeira grapes expressed as absorbance at 520 nm per g of freeze-dried (DW) material.

	Mean ± SD	Minimum	Maximum
Control	0.21 ± 0.042	0.17	0.26
Infected	0.13 ± 0.038	0.05	0.17

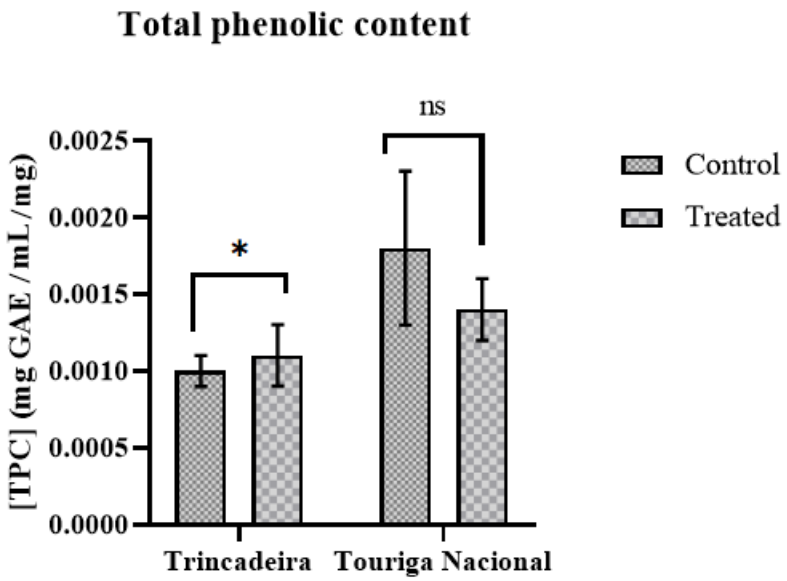
[a]Values expressed are mean ± SD (standard deviation).



Appendices, Figure 6 – Agarose gel electrophoreses showing the assessment of four RNA samples integrity from Trincadeira controls and infected with *Botrytis cinera*. Purified RNA was run in 1.1% agarose gel in 1 x TAE buffer. The agarose gel contained 3 µL of GelRed® nucleic acid gel stain. Samples on left are non-infected (control) and on right are infected.

Appendices, Table 8 - RNA quantification after extraction and purification. RNA was obtained from harvest stage (EL38) of Trincadeira grapes.

Sample name	**Concentration (ng/µL)**	**A_{260}/A_{280}**	**A_{260}/A_{230}**
Control 1	618.5	2.09	2.27
Control 2	342.4	2.10	2.27
Control 3	778.5	2.13	2.29
Control 4	603.7	2.12	2.25
Infected 1+2	454.3	2.06	2.21
Infected 3+4	893.2	2.15	2.33
Infected 5+6	262.6	2.09	2.30
Infected 7	328.7	2.09	2.31



Appendices, Figure 7 – Total phenolic content (TPC) as gallic acid equivalent (mg GAE / mL) per mg of lyophilized grapes of controls and treated Trincadeira samples with melatonin. Results are expressed as mean ± standard variation (SD). Data were analysed with Student´s t- test ($P < 0.05$): for each compound, 'ns' means none significant ($P > 0.05$) difference between the different samples, while '*' indicates significant differences between samples ($P \geq 0.01$ to 0.05).

Appendices, Table 9 – Weight values (g) of Trincadeira and Touriga Nacional lyophilized grapes with and without melatonin used in quantification of phenolic content.

		Weight (g)	
	Samples	Trincadeira	Touriga Nacional
Controls	C1	0.0261	0.0282
	C1 r	0.0241	0.0235
	C2	0.025	0.0261
	C2 r	0.026	0.0256
	C3	0.0247	0.0208
	C3 r	0.0204	0.0293
	C4	0.0253	0.02
	C4 r	0.0224	0.0298
Treated	T1	0.028	0.0256
	T1 r	0.024	0.0275
	T2	0.0267	0.0205
	T2 r	0.0236	0.0213
	T3	0.0234	0.0269
	T3 r	0.0245	0.0244
	T4	0.0257	0.0257
	T4 r	0.028	0.024

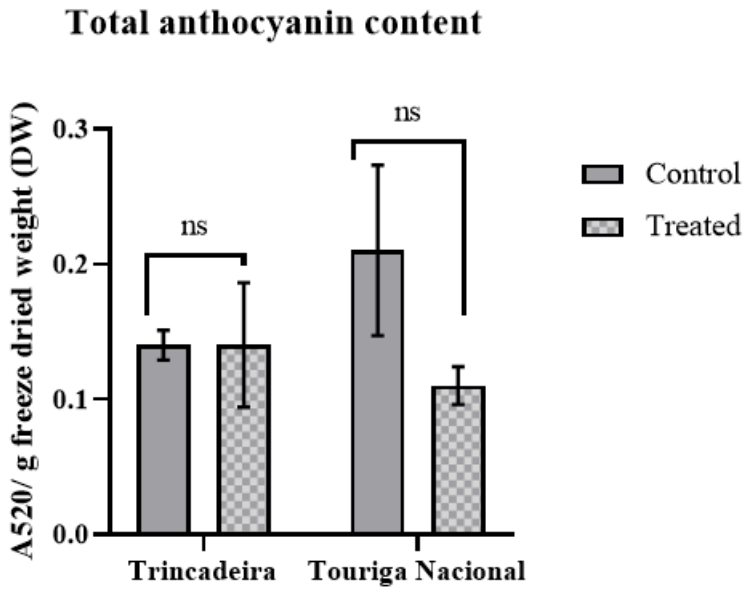
Appendices, Table 10 - Total phenolics content (TPC) as gallic acid equivalent (mg GAE / 100 mL) per mg of lyophilized grapes of Touriga Nacional and Trincadeira samples controls and with melatonin treatment[a] at the harvest stage (EL38).

			Mean ± SD	Minimum	Maximum
Water	Touriga Nacional	Controls	0.0018 ± 0.0005	0.0013	0.0027
		Treatment	0.0014 ± 0.0002	0.0012	0.0017
	Trincadeira	Controls	0.0010 ± 0.0001	0.0008	0.0012
		Treatment	0.0011 ± 0.0002	0.0010	0.0015

[a]Values expressed are mean ± SD (standard deviation) of two experiments.

Appendices, Table 11 – Weight values (g) of Touriga Nacional and Trincadeira lyophilized grapes with and without melatonin used in quantification of anthocyanins.

		Weight (g)	
	Samples	Touriga Nacional	Trincadeira
Controls	C1	0.0516	0.0501
	C2	0.0499	0.0511
	C3	0.0493	0.0496
	C4	0.0502	0.05
Treated	T 1	0.0507	0.0499
	T 2	0.0503	0.0505
	T 3	0.0494	0.0496
	T 4	0.0515	0.0493



Appendices, Figure 8 – Total anthocyanin content expressed as absorbance at 520 nm per g of freeze-dried (DW) material of controls and treated Trincadeira samples with melatonin from Trincadeira and Touriga Nacional samples. Results are expressed as mean ± standard variation (SD). Data were analysed with Student´s t- test ($P < 0.05$): for each compound, 'ns' means none significant ($P > 0.05$) difference between the different samples.

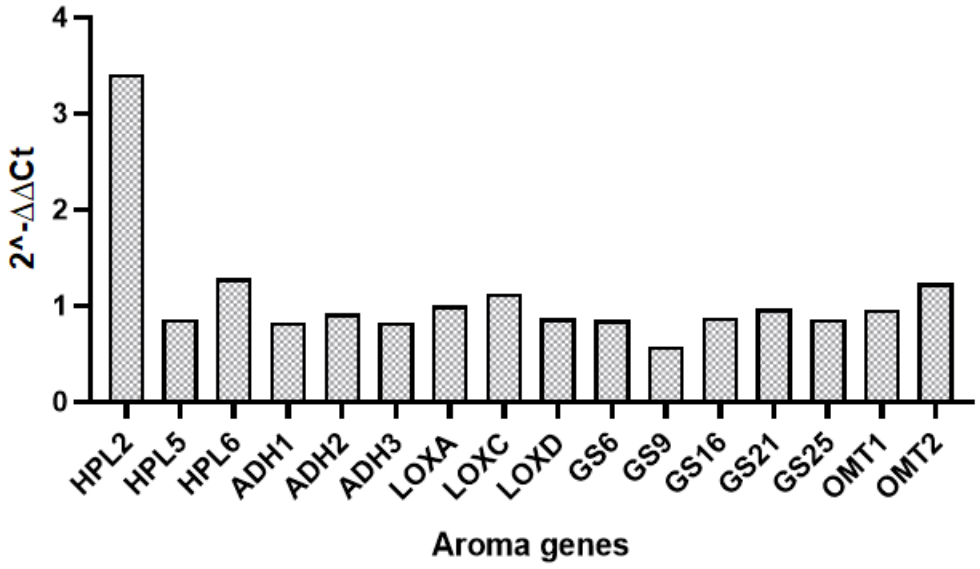
Appendices, Table 12 - Total anthocyanin content of controls and treated[a] Trincadeira with melatonin samples expressed as absorbance at 520 nm per g of freeze-dried (DW) material.

		Mean ± SD mg/mL	Minimum	Maximum
Trincadeira	Control	0.14 ± 0.011	0.12	0.15
	Treated	0.14 ± 0.046	0.07	0.19
Touriga Nacional	Control	0.21 ± 0.063	0.13	0.29
	Treated	0.11 ± 0.014	0.09	0.13

[a]Values expressed are mean ± SD (standard deviation).

Appendices, Table 13 – Expression of delta delta Ct (ΔΔCt) and expression fold change (2^-ΔΔCt) values from the difference between non-infected (control) and infected from all the genes (primer) Ct against reference genes Ct (in this case with elongation factor). Similar results were obtained with actin.
Expression is plotted as: CT gene – Ct elongation factor. High Ct values indicate high gene expression and vice versa.

Genes (primer) ID	ΔΔCt	2^-ΔΔCt
HPL2	-2.0224	4.0627
HPL5	0.1174	0.9219
HPL6	0.2340	1.17612
ADH1	0.3810	0.7679
ADH2	0.1157	0.9229
ADH3	0.2483	0.8419
LOXA	0.0208	0.9857
LOXC	-0.4572	1.3729
LOXD	-0.2597	1.1972
GS6	0.2021	0.8693
GS9	0.5970	0.6611
GS16	0.2072	0.8662
GS21	0.0851	0.9427
GS25	0.2709	0.8280
OMT1	-0.0347	1.0244
OMT2	-0.7035	1.6284



Appendices, Figure 9 – Bar plot of 2^-ΔΔCt expression fold change results from each gene studied. The fold change in expression of the several target gene (*HPLs, ADH, LOX, GS, OMT*) relative to the internal control gene (elongation factor) at harvest stage (EL38). The samples were analysed using real-time quantitative PCR (RT-qPCR) and the Ct data were imported into Microsoft Excel. LinRegPCR was the program used to calculate the Ct from each sample. The mean fold change in expression of the target gene at harvest stage was calculated using the Ct (target gene) – Ct (elongation factor) equation, to non-infected (control) and infected, where -ΔΔCt was calculated Ct (infected) – Ct (non-infected). The 2^-ΔΔCt was calculated after knowing the -ΔΔCt result.